Characterisation of Arcuate Nucleus Projections to the Paraventricular Nucleus of Thalamus

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Abstract

The metabolic state (fed or hungry) has been shown to influence varying responses to food-associated cues. Recent studies reveal that the paraventricular nucleus of the thalamus (PVT) may play a critical role as it receives inputs from the hypothalamic arcuate nucleus (ARC), a major regulator of appetite and energy homeostasis. The PVT also relays outputs to the nucleus accumbens, known to mediate behavioural responses to reward-associated cues.

The ARC receives information about metabolic state via hormones from the periphery, but it is unknown if this information is transmitted to the PVT. This study investigated leptin, a key metabolic hormone penetrating the ARC, which signals the brain to modulate food intake. Produced in adipose tissue, leptin suppresses food intake and stimulates metabolic processes to dissipate excessive energy stores; with intrinsic levels being low in hungry states. However, it is not known how or if the leptin signal in ARC is transmitted to the PVT. To address this question, I investigated whether ARC neurons projecting to the PVT respond to leptin. Subsets of this goal included mapping the location of these neurons in the ARC; and evaluating their response to leptin. I hypothesized that these ARC neurons express leptin markers.

For this purpose, male Wistar rats (n = 11) were injected with a GFP (green fluorescent protein)-expressing retrograde viral vector (AAVrg-Syn-ChR2(H134R)-GFP) in the PVT. A subset of rats (n = 7) were later maintained on food restriction and injected with leptin (80µg/kg s.c.) 90 minutes before perfusion. Using immunohistochemical staining for GFP, coronal brain sections (20/rat; 40µm thickness) through the extent of the ARC were examined and every retrogradely-labelled GFP cell quantified, mapped and correlated with the injection spread in the PVT. In leptin-injected rats, double immunohistochemistry was performed for GFP and pSTAT3 (phosphorylated signal transducer and activator of transcription 3), a downstream signalling marker for leptin.

Results showed that in 9 rats, the injection site clearly involved PVT, and this correlated with the presence of GFP-labelled neurons in the ARC. Furthermore, these GFP-labelled, PVT-projecting neurons (n = 150) were mostly located in the medial (n = 74) and lateral ARC (n = 61) with some cells located in the dorsal ARC (n = 15). Interestingly, in leptin-treated rats co-localization of GFP cell bodies with pSTAT3-labelled nuclei occurred in ~one-third of GFP-labelled ARC neurons (n = 21/64), indicating that a sizable proportion of ARC neurons with projections to PVT respond to the key metabolic hormone leptin, thus confirming my hypothesis. These co-labelled neurons were located only in the medial (n = 15) and lateral ARC (n = 6), completely excluding the dorsal ARC. This distribution is consistent with that of ARC neurons expressing the feeding-related neuropeptides, NPY/AgRP (neuropeptide Y and agouti-related peptide) and POMC/CART (proopiomelanocortin and cocaine-and-amphetamine-regulated transcript).

This ARC-PVT pathway is potentially important for regulating responses to food cues according to metabolic state. Future studies are needed to determine if neurons in the PVT that receive this input are involved in integrating it with reward signal information.

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3V	Third ventricle
AAV	Adeno-associated virus
AP	Anteroposterior
AgRP	Agouti-related peptide
AMG	Amygdala
ARC	Arcuate nucleus of hypothalamus
ArcD	Dorsal ARC
ArcL	Lateral ARC
ArcM	Medial ARC
BSA	Bovine serum albumin
CART	Cocaine-and-amphetamine-regulated transcript
CCK	Cholecystokinin
ChR2	Channelrhodopsin-2
CNS	Central nervous system
DA	Dopamine
DAPI	4′,6-diamidino-2-phenylindole
DM	Dorsomedial hypothalamic nucleus
EDTA	Ethylenediaminetetraacetic acid
GABA	γ-aminobutyric acid
GFP	Green fluorescent protein
GLP-1	Glucagon-like peptide 1
HPC	Hippocampus
IL-6	Interleukin-6
JAK2	Janus kinase 2

LH	Lateral hypothalamus
MCR	Melanocortin receptor
NAc	Nucleus accumbens
NGS	Normal goat serum
NPY	Neuropeptide Y
Ob-Rb	Leptin receptor
PB	Phosphate buffer
PBS	Phosphate buffer saline
PFA	Paraformaldehyde
PFC	Prefrontal cortex
POMC	Proopiomelanocortin
pSTAT3	Phosphorylated signal transducer and activator of transcription 3
PVT	Paraventricular nucleus of thalamus
PVN	Paraventricular nucleus of hypothalamus
s.c.	Subcutaneous
STAT3	Signal transducer and activator of transcription 3
Syn	Synapsin
VTA	Ventral tegmental area

1. Introduction

Our metabolic state, i.e. hungry or satiated, has been shown to influence varying behavioural responses to food-associated cues. This regulation of food intake is thought to be mediated by an interrelated and integrated communication between homeostatic metabolic systems and non-homeostatic reward systems (Ferrario et al., 2016; Matafome and Seica, 2017; Saper et al., 2002; Volkow et al., 2011). However, the neural circuitry and mechanisms underlying this complex process has not been fully elucidated. The main purpose of the present study was to investigate a recently identified neural circuit which potentially integrates homeostatic metabolic signals with the non-homeostatic circuit to drive reward-mediated feeding. In this introduction, I will review the background to this investigation by firstly reviewing the homeostatic control of food intake, the platform on which non-homeostatic factors are superimposed. Secondly, I will review the non-homeostatic control of feeding and the reward system. The third part will introduce a critical circuit through the paraventricular nucleus of the thalamus (PVT), and its possible role in the integration of homeostatic and non-homeostatic systems. Finally, I will summarise the specific questions that I aim to address with this study.

1.1 Homeostatic Regulation of Food Intake

The homeostatic regulation of food intake is characterised by physiological processes that control energy balance and body weight by increasing or decreasing eating motivation and energy expenditure following fluctuations of energy stores (Lutter and Nestler, 2009; Schwartz et al., 2000). This regulation is mediated by short-term feedback to ensure that amounts of food ingested can be accommodated; by longer-term feedback to ensure energy stores are adequate; and by cues from the environment to promote eating when food is obtainable (Andermann and Lowell, 2017; Lutter and Nestler, 2009). This complex system involves orexigenic (appetite-stimulating) and anorexigenic (appetite-suppressing) peripheral signals from the

gastrointestinal tract, adipose tissue and pancreas- which informs the brain of our nutritional status. Brain networks in the central nervous system (CNS) which monitor, process and integrate this information then coordinate the metabolic responses and ingestive behaviours needed to correct any macronutrient imbalance (Berthoud et al., 2017; Hopkins et al., 2016; Matafome and Seica, 2017).

Gut, brain and adipose tissue-derived peptides are critical determinants of both the metabolic responses to food and the initiation of food intake (Chen, 2016; Reichelt et al., 2015). These appetite-controlling signals include hunger peptides such as orexin and ghrelin, which induce food intake; and satiety signals like cholecystokinin (CCK), glucagon-like peptide 1 (GLP-1) insulin and leptin- which inhibit food intake (Chen, 2016; Yu and Kim, 2012). This study will be focused on leptin.

1.1.1 Role of Leptin in Homeostatic Control of Food Intake

Leptin is an important metabolic hormone involved in the regulation of food intake and has been one of the most influential discoveries for obesity research in the last two decades (Berthoud, 2006; Klok et al., 2006; Lutter and Nestler, 2009). Leptin is secreted by adipose tissue and plays a key role in both long and short-term regulation of energy balance by relaying information about peripheral energy levels to the brain (Monteleone and Maj, 2013).

On the one hand, in the long-term regulation, leptin plays a chronic role in body weight homeostasis and metabolic role in the choice of fuels and homeostasis of energy storage to be utilised according to the body's needs (Atelle et al., 2002; Blundell et al., 2001; Saladin et al., 1995). During states of positive energy balance, the release and circulating levels of leptin are increased proportional to fat stores where it acts centrally, constraining fat mass by reducing food intake and stimulating metabolic processes to dissipate excessive energy stores- supporting energy expenditure and weight loss (Guyenet and Schwartz, 2012; Liu and Kanoski,

2018; Lutter and Nestler, 2009; Monteleone and Maj, 2013). Conversely, during states of negative energy balance, adipose mass contracts and levels of leptin fall, which then favours increased food intake and decreased energy expenditure to conserve limited fuel stores (Ahima et al., 1996; Woods et al., 1998).

On the other hand, the short-term regulation of leptin has been recently discovered to involve the release of leptin from the stomach that contributes to short-term satiation after a meal. The acute control of feeding by gastric leptin is sensitive upon nutritional status, being rapidly mobilized in response to food intake following fasting (Attele et al., 2002; Pico et al., 2003). This can be supported by subsequent studies which showed that leptin levels in the blood increase when animals are fed and fall when animals are deprived of food (Saper et al., 2002). Thus, the acute intrinsic levels of leptin are low in a hungry state and high in a satiated state (Chen, 2016; Hussain and Khan, 2017; Klok et al., 2006; Saper et al., 2002). My project focused on this short-term signalling of leptin.

The homeostatic actions of leptin are mediated mainly in the hypothalamus of the CNS, where it crosses the blood-brain barrier and binds onto its leptin receptor, Ob-Rb, located on the cell membrane of neurons. Ob-Rb is an interleukin-6 (IL-6)-type class 1 cytokine receptor and is expressed in several hypothalamic regions, including the ventromedial, dorsomedial and arcuate nuclei (Ingvartsen and Boisclair, 2001; Saper et al., 2002). However, studies using circulating radiolabelled leptin indicate that the densest expression is within neurons in the arcuate nucleus (ARC) (Ingvartsen and Boisclair, 2001; Saper et al., 2002; Yu and Kim, 2012). Binding of leptin to the receptor then induces the activation of the Janus kinase 2 (JAK2)- signal transducer and activator of transcription 3 (STAT3) signalling pathway (Yu and Kim, 2012). Detection of STAT3 activation through labelling of its phosphorylated form is a key marker of leptin signalling, which I used in the project (Munzberg et al., 2003).

1.1.2 Homeostatic Action of Leptin in the ARC

The ARC, located adjacent to the ventral part of the third ventricle, is a brain region critical for the regulation of food intake and is the primary area for the action of leptin in the CNS (Baver et al., 2014). The ARC's proximity to the third ventricle paired with its modified, less restricted blood-brain barrier increases its sensitivity to circulating signals pertaining to energy status. This allows nutrient signals like leptin to directly diffuse into the extracellular fluid, giving the ARC a major role in feeding control circuits (Chen, 2016; Waterson and Horvath, 2015). Previous studies which showed that the ARC transduces information related to leptin signalling into a neuronal response include the anorexic response to local microinjection of leptin in this area and the inability of intracerebroventricular leptin to reduce food intake after the ARC has been destroyed (Dawson et al., 1997; Tang-Christensen et al., 1999).

The effects of leptin are mediated in particular through interactions with the central melanocortin system, which consists of several neuronal populations and have been listed as key players in the ARC and in the regulation of energy homeostasis (Ellacott and Cone, 2006; Cone, 2005). The main components of the central melanocortin system are summarised in Figure 1.1. and are 1) the proopiomelanocortin (POMC) and cocaine-and-amphetamine-regulated transcript (CART)-co-expressing neurons (POMC/CART) located in the lateral ARC, 2) the neuropeptide Y (NPY) and agouti-related peptide (AgRP)-co-expressing neurons (NPY/AgRP) located in the medial ARC, and 3) the melanocortin receptors (MCR) expressed in second-order neurons located in the paraventricular nucleus of the hypothalamus (PVN) and lateral hypothalamus (LH) (Chen, 2016; Schwartz et al., 2000; Yu and Kim, 2012).

The components of the central melanocortin system located in the ARC are important in mediating the effects of leptin. The POMC/CART co-expressing neurons, located in the lateral ARC, are revealed to suppress feeding; conversely, the NPY/AgRP co-expressing neurons, located in the medial ARC, are known to promote feeding (Suzuki et al., 2010; Takahashi and

Cone, 2005; Wang et al., 2015). Leptin has been shown to stimulate these anorexigenic POMC/CART co-expressing neurons and inhibit the orexigenic NPY/AgRP co-expressing neurons, which can then lead to the overall depression of appetite and weight loss (reviewed in Liu and Kanoski, 2018; Figure 1.1). There is extensive evidence to support this. Firstly, Cheung et al. (1997) found that leptin receptors are expressed on the majority of POMC neurons in the ARC. Secondly, following leptin administration, the expression of phosphorylated signal transducer and activator of transcription 3 (pSTAT3), a protein downstream of the leptin receptor signalling cascade was upregulated in POMC neurons of the ARC (Elias et al., 1999; Munzberg et al., 2003). And finally, in an *ex vivo* electrophysiological slice preparation in rodents, leptin was shown to alter the firing rate of ARC POMC and NPY/AgRP neurons (Cowley et al., 2001; Takahashi and Cone, 2005). These findings indicate that the central melanocortin system is downstream of leptin receptor signalling and plays a key role in mediating the effects of this adipostatic (produced in adipose tissue and negatively regulates feeding) hormone.

In summary, the homeostatic control of feeding involves peripheral signals from appetite-controlling peptides, like leptin. We can highlight the central role of the ARC as a forebrain substrate critically involved in the homeostatic regulation of food intake. The ARC coordinates the metabolic responses to the peripheral signals, which can then trigger feeding behaviours to maintain energy homeostasis.

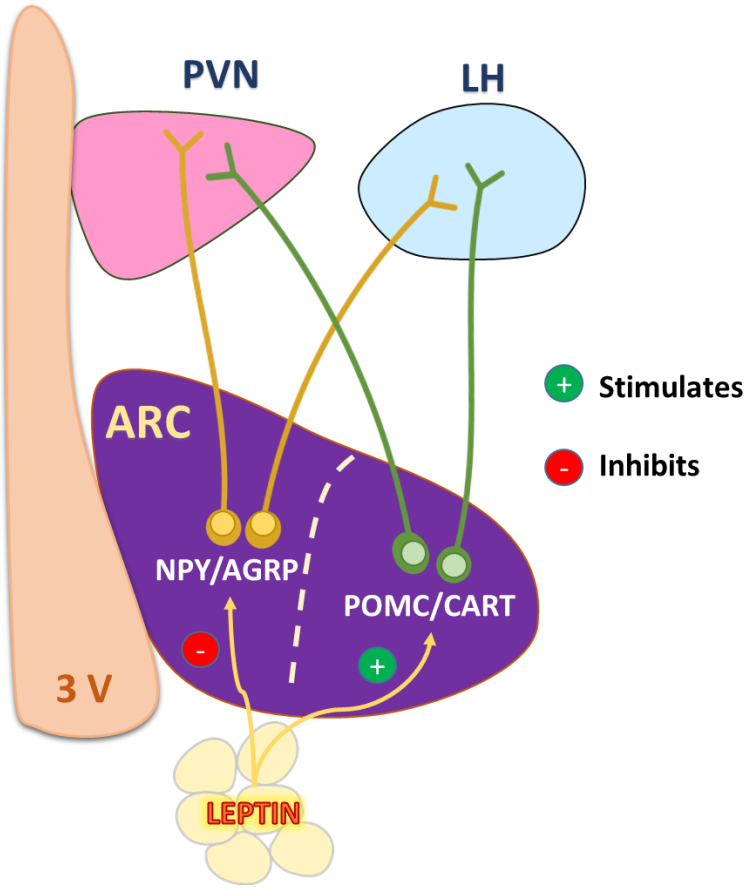

Figure 1.1 Schematic representation of the cell types and connections engaged by leptin in the ARC. Diagram shows known interactions of leptin with specific neural phenotypes in the arcuate nucleus (ARC) with projections to structures important for homeostatic regulation of food intake. (-), (+) indicate inhibitory or stimulatory effect. POMC/CART: proopiomelanocortin and cocaine-and-amphetamine-regulated transcript co-expressing neurons. NPY/AgRP: neuropeptide Y and agouti-related peptide co-expressing neurons. PVN: paraventricular nucleus of hypothalamus. LH: lateral hypothalamus. 3V: third ventricle.

1.2 Non-Homeostatic Regulation of Food Intake

In addition to the internal hormonal signals that drive homeostatic regulation of food intake, external (environmental) cues and psychological processes have also been shown to play an important role in food consumption (Berthoud, 2006; Hernandez Ruiz de Eguilaz et al., 2018; Higgs et al., 2017). These external factors that drive food intake exert their effects on subjective feelings of satiety and hunger can lead to food intake that is non-homeostatic, i.e. not regulated

by actual metabolic needs. Non-homeostatic feeding is associated with motivational behaviours activated by the neuronal reward system in response to any highly palatable food, i.e. any food which independently of its nutritional value produces a pleasurable sensation (Hernandez Ruiz de Eguilaz et al., 2018; Mela, 2006). When eating food that evokes a pleasurable response, we will come to associate the characteristics of that food (e.g. the sight and the smell of the food) with the positive consequence ('liking' response). As a result of this learning, the food-associated visual and olfactory cues acquire the ability to become sought after (they become 'wanted') (Berridge, 1996). For example, we might have a strong desire to consume pizza if we see a shop advertising pizza from which a strong smell of pizza is emanating. To support this, it was previously shown that highly attractive food cues promote food-seeking, which drives impulse buying, particularly that of high-calorie food (Berridge, 1996; Nisbett et al., 1969; Saper et. al., 2002; Tal and Wasink, 2013). Thus, this demonstrates that purchasing and consumption behaviours are strongly influenced by the reward value of food cues.

Such non-homeostatic mechanisms can be powerful enough to override the homeostatic regulation of food intake. For example, food-associated stimuli provoke consumption of highly palatable food of high energy density, even when sated; or conversely, in anorexia, individuals can forgo eating despite severe energy deficit (Ferrario et al., 2016; Lutter and Nestler, 2009). "Reward systems" of the non-homeostatic pathway that influence motivational processes are thought to be capable of overriding physiological hunger and satiety cues to influence these reward-mediated feeding behaviours (Liu and Kanoski, 2018). Until recently, most studies have focused on the role and neural mechanisms of the homeostatic aspect of appetite regulation. However, due to the current increased prevalence of obesity and eating disorders such as anorexia worldwide, interest in understanding how animals and humans eat in a non-homeostatic manner, beyond metabolic needs, has become a priority in recent years (Alonso-Alonso et al., 2015; Yu and Kim, 2012).

Identifying the neural circuitry and mechanisms responsible for the rewarding properties of food has significant implications on understanding energy balance and the development of obesity and anorexia. Through experiments performed in rodents and humans, there has been considerable evidence revealing that the reward-mediated behaviours associated with the consumption of highly palatable foods converge on the mesolimbic dopamine (DA) pathway (Alonso-Alonso et al., 2015; Lutter and Nestler, 2009). Understanding the mechanisms by which the DA reward system and the homeostatic pathway interact will be important for defining the brain mechanisms underlying non-homeostatic feeding.

1.2.1 Mesolimbic Dopamine Pathway

The mesolimbic DA pathway or the reward pathway, consists of DA-releasing neurons from midbrain ventral tegmental area (VTA) which project to the medial prefrontal cortex (PFC), amygdala (AMG), hippocampus (HPC) and nucleus accumbens (NAc) (Ferrario et al., 2016; Matafome and Seica, 2017; Yu and Kim, 2012; Figure 1.2). The NAc is a central node for reward processing known to mediate arousal, motivation and reward behaviours (Li et al., 2016; Parsons et al., 2006).

Previous *in vivo* brain microdialysis studies indicated that DA release in the NAc significantly increases during consumption of a palatable meal (Hernandez and Hoebel, 1988; Wilson et al., 1995). Additionally, DA has been shown to be involved in the control of conditioned incentive motivation, as conditioned cues associated with food reward could evoke NAc DA release (Liu and Kanoski, 2018). These studies show that dopaminergic input to the NAc from the VTA encodes the conditioned rewarding properties food and that these rewarding aspects further enhance incentive salience and motivation to acquire of palatable foods.

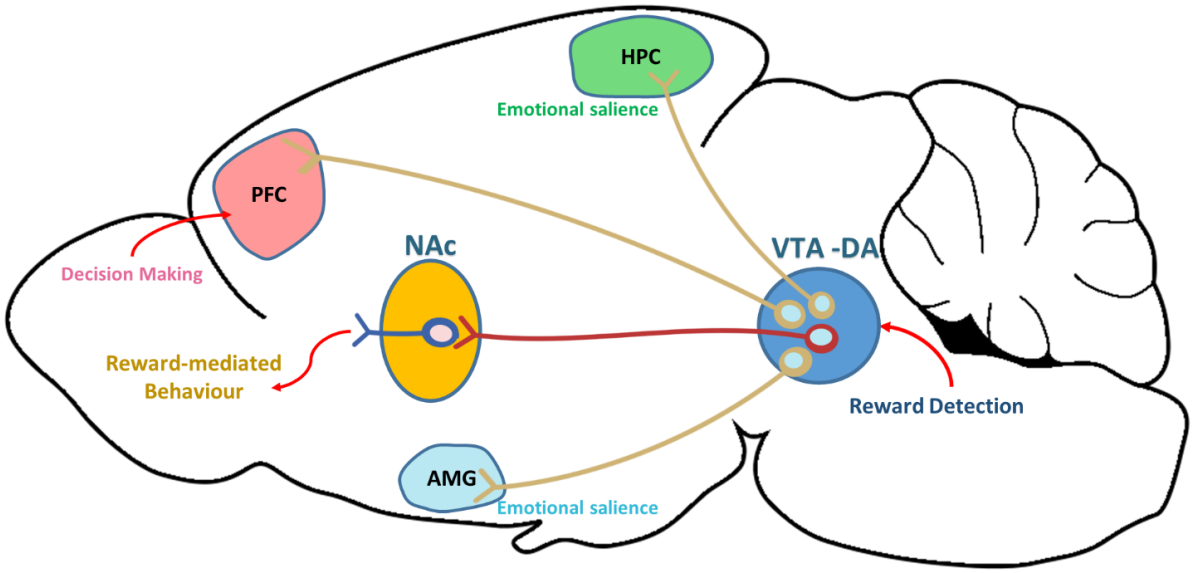

Figure 1.2 Schematic of simplified mesolimbic dopamine pathway in the rodent brain. Red neuron represents dopaminergic projection from the ventral tegmental area (VTA) to the nucleus accumbens (NAc) which mediates reward-associated behaviours. Yellow neurons represent other dopaminergic projections to limbic and cortical sites that regulate emotion, cognition and learning (Fulton, 2010). DA: dopamine. HPC: hippocampus. PFC: prefrontal cortex. AMG: amygdala.

1.3 Possible Role of the PVT in the Integration of Homeostatic and Non-homeostatic Systems

Previously, the mechanisms of homeostatic and non-homeostatic regulations of food intake were investigated somewhat separately. However more recently, it has been specially proposed that these two systems integrate through neurochemical crosstalk (Berthoud, 2006; Berthoud et al., 2017). This approach is consistent with incentive motivation theories of behaviour, which argue that our metabolic state, i.e. hungry or satiated, influences eating behaviour by modulating the hedonic value of food and food-associated cues (Toates, 1986). For example, food is more highly liked and desired when hungry and less liked when satiated: the smell and taste of a pizza is usually less alluring when we have just eaten (Berridge et al., 2010). Experiments performed in rodents also showed that both food restriction and acute food

deprivation enhances the rewarding effects of food (Fulton et al., 2010). However, the important question to address is where and how do these metabolic and non-metabolic factors integrate to induce food intake? This thesis is investigating one of the possible sites of such integration, i.e. the paraventricular nucleus of the thalamus (PVT).

There are several lines of evidence for the interaction between homeostatic and non-homeostatic systems. Firstly, rodent and human studies have revealed that hedonic feeding is modulated by peripheral metabolic signals, like leptin, that are involved in the homeostatic control of energy balance (Fulton, 2010; Matafome and Seica, 2017). Leptin has been shown to influence the mesolimbic DA system by acting on LH neurons, which project to the VTA and influence reward-seeking behaviours (Fulton, 2010; Kiefer et al., 2001). This indirect peptide activity of leptin on the mesolimbic DA pathway may provide a regulatory link between energy homeostasis and reward-related behaviour.

Secondly, the ARC has also been recently shown to be modulated by sensory and hedonic information to mediate conditioned aspects of appetite (Liu and Kanoski, 2018). This is evident by findings that the activity of AgRP and POMC neurons are regulated not only by energy status but also by food palatability (Chen et al., 2015). Additionally, using deep-brain calcium imaging, Betley et al. (2015) revealed that the activity of AgRP neurons not only rapidly reduced in response to feeding but also following exposure to conditioned food-related cues. Thus, the ARC, which is classically linked with homeostatic food intake control, also plays an important role in conditioned and hedonic aspects of feeding behaviour.

In addition to the pathways reviewed above, there is a growing body of evidence which suggests that the thalamic midline nuclei may pose as an important integrative function for reward-mediated behaviour (Li et al., 2016). This project focused on the PVT. Therefore, the next section presents a detailed review of the role of the PVT in reward and food intake and its

potential implication as a critical node linking homeostatic and non-homeostatic regulations of feeding.

1.3.1 Role of PVT in Reward

The midline thalamus consists of dorsal, ventral and posterior groups of nuclei. Most is known about the dorsal group, and especially the PVT, located in the dorsal midline of the thalamus directly underneath the third ventricle. The PVT has been suggested to play a critical role in mediating behavioural responses to reward-associated cues (Haight et al., 2015; Haight et al., 2017; Lee et al., 2015; Van der Werf et al., 2002). Such reward functions are implicated by numerous connections of the PVT with cortical and limbic areas that mediate motivated behaviours. These are summarized in Figure 1.3. Areas that are innervated by the PVT include the prelimbic cortex, infralimbic cortex, PFC, extended AMG, and the ventral subiculum, an area of the HPC involved in modulating both emotional and motivated behaviours (Kirouac, 2015). Conversely, the PVT also receives reciprocal inputs from these cortical and limbic areas (Kirouac, 2015). Thus, the PVT is critically placed in an anatomical position to influence key forebrain circuits believed to contribute to reward-mediated behaviours.

Additional evidence supporting a role for the PVT in mediating the propensity to attribute incentive salience to reward cues comes from its association with the DA reward pathway. Firstly, several retrograde tracing studies demonstrated that the PVT sends robust outputs to the NAc, a brain area known to control motivated behaviour (Kelley et al., 2005; Kirouac, 2015; Li and Kirouac, 2008). Second line of evidence which supports this association was that the PVT has been shown to modulate dopamine neuron activity. Following chemogenetic activation of the PVT in rats, Perez and Lodge (2018) reported that the number of spontaneously active DA neurons in the VTA increased with PVT inactivation reversing aberrant DA neuron activity. Finally, a number of studies revealed that the PVT is highly active during periods of

arousal in rats and electrical stimulation of the PVT increased DA levels in the NAc, and the mechanism for this release appears to involve activation of presynaptic ionotropic glutamate receptors in DA fibres (Haight et al., 2017; Parsons et al., 2007). These examples indicate that modulation of VTA-DA levels in the NAc by the glutamatergic projections from the PVT may be linked to arousal-induced increases in DA tone and could be involved in the facilitation of specific behavioural patterns associated with motivation and reward.

1.3.2 Role of PVT in Food Intake

Importantly, much of the research surrounding the PVT and reward-seeking behaviours has focused on the role of the PVT in the regulation of food intake. The PVT has been implicated in behavioural arousal triggered by visceral state. Lesions of the PVT or inactivation of the posterior PVT with the GABA (γ- aminobutyric acid) agonist muscimol was shown to increase food intake in rats and attenuate increases in locomotor activity (Bhatnagar and Dallman, 1999; Stratford and Wirtshafter, 2013). Additionally, using cFos as a marker of neuronal excitation, Igelstrom et al. (2010) showed that cFos induction in the PVT increased following the presentation of cues that predicted delivery of a sucrose solution. Furthermore, the PVT was also found to be activated when rats are placed in a context that had been previously paired with palatable food, whereas placing rats in a context paired with regular chow did not increase neural activity in the PVT (Choi et al., 2010). These experimental evidences, therefore, indicate that the PVT may contribute to food intake in situations involving high levels of arousal including by food-deprived states or in conditions involving palatable food.

Several retrograde tracing studies have also identified that the brainstem nuclei, prominently associated with processing somatosensory and visceral information, provide a significant input to the PVT (Kirouac et al., 2006; Li and Kirouac, 2012; Otake and Ruggiero, 1995; Figure 1.3). Inputs from the prelimbic and infralimbic cortices to the PVT have also been shown to play an

important role in the integration of physiological states with salient environmental cues to guide behaviour (Li and Kirouac, 2012; Kirouac, 2015). Interestingly, the PVT also receives an especially strong input from hypothalamic areas involved in the circadian and homeostatic regulation, including a very dense innervation from neuropeptide containing neurons (Kirouac et al., 2006; Lee et al., 2015; Otake, 2005; Van der Werf et al., 2002). Hypothalamic areas that have been identified as sources of inputs to the PVT include the dorsomedial nucleus, suprachiasmatic nucleus, ventromedial nucleus, PVN, and of particular relevance to the present study, the ARC (Li and Kirouac, 2012). These anatomical relationships, in combination with functional studies, allow me to propose that the PVT is a structure of interest and potentially plays a key role as an interface between homeostatic energy-related hypothalamic signalling from the ARC and high-level functions from reward-mediating brain areas of the mesolimbic DA system.

1.3.3 The Link Between the PVT and ARC

There has been an emerging interest in the linkage between the ARC and PVT and their association in the hedonic behaviours of feeding according to metabolic state. It is known that the regulation of energy balance in the ARC involves anorexigenic POMC/CART co-expressing neurons in the lateral ARC, as well as orexigenic NPY/AgRP neurons located in the medial ARC. Previously, these internally oriented sensory neuropeptides that read circulating metabolic signals of physiological energy state, were shown to project to the PVN (Cone, 2005). However, interestingly, these different neuronal populations have also recently been shown to project to the PVT.

There are several anatomical studies which demonstrated the connection between the ARC and the PVT. Koylu et al. (1998) initially reported that the PVT contains a dense plexus of CART fibres. Through immunofluorescence and retrograde tracing experiments, these CART-expressing neurons in the PVT were shown to originate from the ARC (Kirouac et al., 2006;

Parsons et al., 2006). Early studies demonstrated that CART negatively regulates feeding behaviour to promote satiety (Kelley et al., 2005). Additionally, through optogenetic stimulation studies, Atasoy et al. (2012) revealed that ARC AgRP neuron projections to the PVT can cause hunger. These PVT innervations of orexigenic and anorexigenic neuropeptides from the ARC elucidates the potential role of the PVT in feeding.

Furthermore, these ARC-PVT projecting neuropeptides have also been shown to project to the mesolimbic reward pathway. PVT neurons that project to the NAc were found to receive interactive POMC/CART and NPY/AgRP terminals, originating from the lateral and medial ARC respectively (Atasoy et al., 2012; Betley et al., 2013; Lee et al., 2015; Parsons et al., 2006). This suggests that ARC projections to the PVT may play an essential role in the integration of metabolic or arousal-related inputs on their way to the mesolimbic reward pathway and may influence motivational and hedonic behaviours in feeding (Kelley et al., 2005; Lee et al., 2015; Parsons et al., 2006; Figure 1.3).

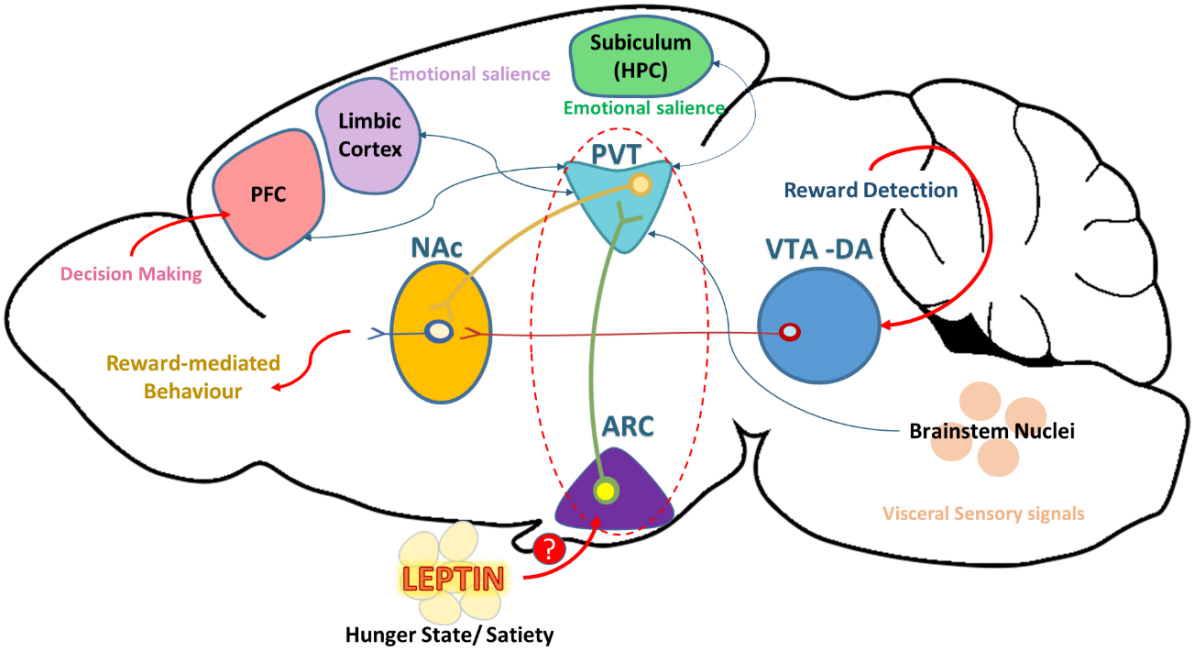

Figure 1.3 Schematic of neural nodes controlling food intake and reward. The PVT may integrate emotional saliency information from the subiculum, limbic cortex and prefrontal cortex (PFC) with viscerosensory information from the ARC and brainstem to guide behaviour. Dotted line indicates the new potentially critical circuit- the projection of ARC neurons to the

PVT and its possible implications in responses to food-associated cues. Question mark denotes an unknown connection between leptin signal in ARC and its transmission to the PVT. PVT: paraventricular nucleus of the thalamus. ARC: hypothalamic arcuate nucleus. NAc: nucleus accumbens. VTA: ventral tegmental area. DA: dopamine. HPC: hippocampus. PFC: prefrontal cortex.

In summary, the above review establishes the following key principles:

- Leptin is a key metabolic signalling hormone, which acts on ARC neurons containing the anorexigenic POMC/CART and orexigenic NPY/AgRP co-expressed neuropeptides
- Neurons with these neuropeptides project to the PVT
- Remains unknown if ARC neurons projecting to the PVT are responsive to leptin

1.4 Aim

The overall aim of this study was to determine whether hypothalamic arcuate nucleus neurons that project to the paraventricular nucleus of the thalamus respond to leptin – a key metabolic hormone that regulates appetite and energy homeostasis.

1.5 Hypothesis

I hypothesized that hypothalamic arcuate nucleus neurons that project to the paraventricular nucleus of the thalamus express leptin activation markers.

1.5.1 Objectives

To test my hypothesis, my specific objectives were:

1. To confirm the **presence** of PVT-projecting neurons in the ARC after injection of a GFP-expressing retrograde viral vector in the PVT
2. **Map** the location of these retrogradely-labelled cells relative to established divisions in the ARC i.e. Are the cells in the ARC medially or laterally located?
3. **Correlate** the presence of cells in the ARC with injection site/spread in the PVT i.e. Is the injection spread in the general area of the PVT sufficient or does it need to be restricted and centred in the PVT?
4. To determine if **leptin** activates retrogradely-labelled neurons using a downstream signalling activation marker i.e. pSTAT3

2. Methods

2.1 Animals and Background Procedures

Prior to the beginning of any experimental work, the proposed project was approved by the University of Otago Animal Ethics Committee.

For my experiments, I used brains from animals which had undergone previous procedures to inject a virus for green fluorescent protein (GFP) expression and had received an injection for leptin or vehicle prior to sacrifice. These procedures were performed by Dr Sonja Seeger-Armbruster, a Post-doctoral Fellow in the Hyland lab, as part of a larger Marsden-funded project investigating how the PVT integrates metabolic signals and reward-seeking behaviours.

Eleven male adult Wistar rats weighing 250-350 g sourced from the University of Otago breeding colony at the Hercus Taieri Resource Unit were used for the study. Animals were housed in environmentally controlled conditions and subjected to an artificial normal 12:12 hour light/dark cycle (light phase: onset at 10 am). They were housed in group cages which allowed socialization with other littermates, minimizing levels of stress (Baumans, 2005). Animals had access to food (standard chow, Teklad Global; 18% Protein Rodent Diet 2918, irradiated; Envigo, USA) and water *ad libitum*. However, a subset of animals (those administered with leptin) were subjected to a reversed 12:12 hour light/dark cycle (light phase: onset at 10 pm). These animals were maintained on a restricted diet regime of 12-15 g rat chow per day (for five days prior to perfusion) and fasted for certain periods (~20 hours prior to perfusion), to minimize intrinsic levels of leptin. This was enough to ensure they remained lean, but with no more than 10% relative reduction in body weight compared to free-feeding animals. The cages were under observation every weekday for at least 10 minutes and animals were handled daily for weighing, human contact habituation, and checking to ensure they maintained a healthy body weight.

2.1.1 Virus Injection

The virus injection procedures are summarized in Figure 2.1. Under general anaesthesia with 2% isoflurane (in a PC2 Bio bubble), stereotaxic methods were used for an intracerebral injection (0.8µL over 8 minutes) of a retrograde adeno-associated viral vector (AAV). An AAVrg-Syn-ChR2(H134R)-GFP vector (genomic titer: 5 x 10e13 gc/ml; Addgene viral prep #58880AAVrg) containing light-activated channelrhodopsin (ChR2) and GFP was injected into the PVT of animals. The viral vector was injected using a Hamilton micro syringe at -2.0 anteroposterior from bregma, midline, and -4.6 dorsoventral from brain surface (Paxinos and Watson, 2009). The virus contained a synapsin (Syn) promoter which allowed specific expression of the construct in neurons. The retrograde nature of this AAV serotype led to synaptic uptake and retrograde transport of the vector to cell bodies of neuronal pathways projecting to the PVT, including those in the ARC, and expression of ChR2-GFP in their terminals in PVT. Post-surgery animals were single-housed for the first week of recovery and then returned to group housing. Any other manipulation, i.e. food restriction, began 3 weeks after this survival surgery.

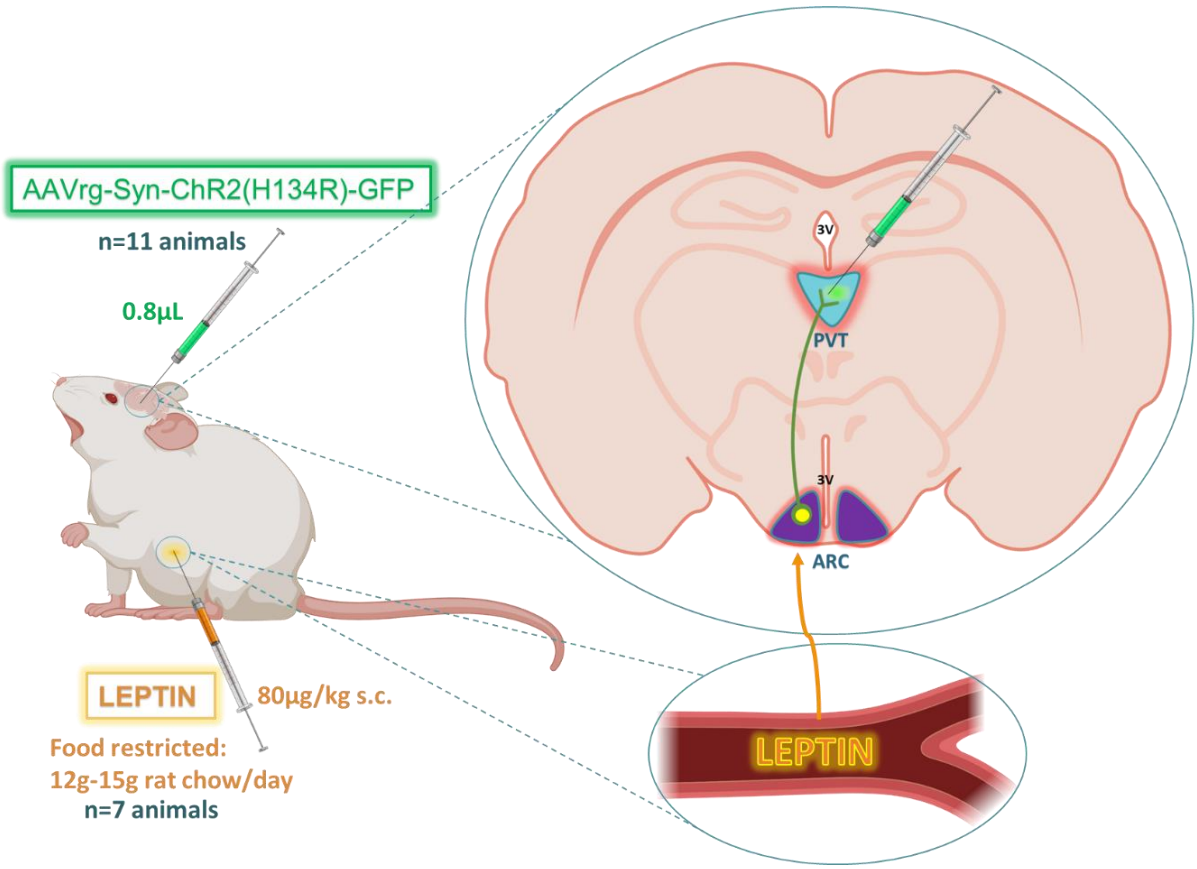

Figure 2.1 Schematic representation of injections administered in Wistar rats. Green neuron indicates retrogradely-labelled neuron in the arcuate nucleus (ARC) with terminals in the paraventricular nucleus of the thalamus (PVT). Yellow dot indicates the nucleus of the neuron. Orange arrow indicates transmission of subcutaneously injected leptin from the bloodstream to the brain. 3V: third ventricle.

2.1.2 Electrophysiology, Leptin Administration and Perfusion

Four to five weeks following the virus injections, animals were re-anaesthetised with 2% isoflurane and underwent acute *in vivo* electrophysiological experiments using stereotaxic methods. An optical fibre for optogenetic stimulation was targeted in the same position as the viral vector injection in the PVT, and a recording electrode was lowered in the ARC to record extracellular action potentials. ARC neurons that project to the PVT were identified by their optogenetic antidromic activation following blue light stimulation (473 nm, intensity 400 mW mm^{-2}) from a laser diode. The data from these experiments are not presented in this thesis.

To detect leptin responsiveness of the neurons in the electrophysiological study and activation of pSTAT3, a sub-group of animals (n = 7) were administered leptin. pSTAT3 is a known activation marker for leptin (Munzberg et al., 2003). Leptin is a hormone responsible for signalling metabolic state associated with being "well-fed" (Hussain and Khan, 2017). To optimise responsiveness to leptin administration, we had to ensure "baseline" or intrinsic levels of leptin were at naturally low levels. To achieve this, leptin animals were maintained on a restricted diet regime of 12-15 g of rat chow per day for the last five days prior to recording experiment and perfusion and were also fasted ~20 hours prior to perfusion. Towards the end of the recording experiment, these animals then received a systemic bolus injection of leptin (80µg/kg, subcutaneous (s.c.); PeproTech; Figure 2.1). This leptin dose has been shown to have physiological effects in the hypothalamus (Cabanelas et al., 2006). Animals were perfused 90 minutes after leptin injection to allow for stable pSTAT3 activation in the ARC. In relation to light/dark cycle, leptin-injected animals were perfused 5-6 hours after light offset; whereas non-leptin-injected animals were perfused 5-6 hours after light onset.

After completion of recordings, animals were deeply anaesthetised with pentobarbital (100mg/kg, intraperitoneal) and the toe-pinch reflex was used to determine deep anaesthesia. Rats were transcardially perfused with 400 ml of 10% sucrose solution (100g sucrose, 1L distilled water; BioFroxx) followed by fixation, 400 ml of 4% paraformaldehyde (PFA; Merck) in 0.1M phosphate buffer (PB; pH 7.2; 40g in 1L of PB; Merck). To maximize fixation, brains were extracted from the skull 2–3 hours after the perfusion, incubated in 4% PFA overnight, and then immersed in 30% sucrose (150g sucrose, 500ml distilled water) using 40mL per brain for at least 48 h for cryoprotection. I received these brains for further processing for my project.

2.2 Histology

I performed the following immunohistochemical procedures and analysis to characterise ARC neurons that project to the PVT as summarised in Figure 2.2.

2.2.1 Sectioning

Coronal brain sections of 40µm thickness were collected using a freezing sliding microtome (Leica SM2400) at -22°C. After fixation of the whole brain section on the microtome stage using 30% sucrose solution, a 20G metal cannula was used to mark the outer edge of one hemisphere of the brain. This needle mark was done to distinguish which hemisphere was left or right on the section once sliced. A wet brush with phosphate buffered saline (PBS; AppliChem), was used to remove the tissue slice off the microtome blade and place it straight into a 24-well plate (Corning® *Costar*®). Tissue slices were stored in cryoprotectant (30mL ethylene glycol, 30g sucrose, 100mL 0.1M PB (pH 7.2)) in the freezer at -20°C until use for immunohistochemistry.

2.2.2 Single GFP Immunohistochemistry

To address my first three objectives of 1) detecting and 2) mapping the location of cell bodies of ARC neurons that project to the PVT and 3) correlating these cell locations with PVT injection size in PVT, single-label immunohistochemistry for GFP was performed. Approximately 20 free floating tissue slices per rat (n = 9) were selected (every 4th section; ~160µm apart) for immunohistochemical analysis. All rinses and incubations occurred on an orbital shaker (Rock-it), set to rotate at 60 rpm. Tissues coming out of cryoprotectant were initially washed 3 x 10 min in PBS and incubated in blocking solution (incubation solution: PBS/0.3% Triton X-100 (Sigma)/0.25% bovine serum albumin (BSA; GIBCO) +5% normal

goat serum (NGS; GIBCO)) to prevent any non-specific binding. This was followed by covered incubation in rabbit anti-GFP primary antibody (1:5000; Life Technologies #a6455) in incubation solution with 2% NGS for 48 h at 4°C. Negative controls were incubated in incubation solution with 2% NGS void of the primary antibody. After 3 x 10 min washes in PBS, tissues (including negative controls) were incubated in a goat anti-rabbit secondary antibody (1:500; AlexaFluor®488; Invitrogen #A-11008) in incubation solution for 2 h at room temperature (20°C) (Figure 2.2). Following a series of washes (3 x 10 min in PBS and 1 x 10 min in PB), tissues were mounted on charged glass slides (Superfrost®Plus) and allowed to dry for ~30 minutes. And finally, slides were coverslipped with Fluoromount-G™ (with DAPI (4′,6-diamidino-2-phenylindole); Invitrogen #E119437) as a mounting medium. After 24 h, coverslips were sealed with nail polish for long term storage.

2.2.3 pSTAT3 and GFP Double Immunohistochemistry

To address my fourth objective of detecting the response to leptin and activation of its downstream signaling marker, STAT3, double immunohistochemistry was performed. I analysed the expression of phosphorylated STAT3 (pSTAT3) in conjunction with GFP after a leptin challenge (n = 7). Similar to the single GFP immuno-protocol, tissues coming out of cryoprotectant were initially washed with PBS (3 x 10 min). Then these tissues underwent additional antigen retrieval for the pSTAT3 protein located in the nucleus, to expose antibody binding site which becomes obstructed during fixation (incubated in 1mM EDTA (Ethylenediaminetetraacetic acid, AppliChem) at 90°C in a water bath; 1x15 min). Following a 3 x 5 min rinse in PBS, tissues were incubated in blocking solution. After another round of washes in PBS (3 x 5 min), sections were incubated in a rabbit anti-pSTAT3 (1:1000; Abcam #ab76315) and chicken anti-GFP primary antibody (1:5000; Aves Labs #GFP-1020) cocktail (in incubation solution + 2% NGS) for 48 h at 4°C. After washes in PBS (3 x 5 min), sections

were incubated in goat anti-rabbit AlexaFluor®568 (1:500; Abcam #ab175695) and goat anti-chicken AlexaFluor®488 (1:500; Abcam #ab150173) secondary antibody cocktail in incubation solution for 2 h at room temperature (20°C) (Figure 2.2). The following washes and mounting procedures were identical to the single-label immunohistochemistry mentioned above.

2.2.4 Microscopy

The staining for GFP-labelled ARC cell bodies, pSTAT3 nuclei and DAPI nuclei were visualised under a fluorescence microscope (Nikon Ti2-E Inverted Widefield Brightfield and Fluorescence). Image overviews of GFP immunoreactivity (automatically stitched montages of 10x images) in sections under the 10x objectives were taken using the NIS-Elements software (2424 x 2424 pixels; 14-bit, ND2 images). Images of the ARC were taken under 10x, 20x and 40x objectives to visualise GFP-labelled (green channel; exposure time:100ms; analog gain: 9.3x) cell bodies, pSTAT3 (orange channel; exposure time: 200 ms; analog gain: 25.6x) nuclei and DAPI (blue channel; exposure time: 100 ms; analog gain: 20.9x) nuclei. The exposure time, gain and illumination parameters emitted by the microscope were kept constant for each antibody and across all samples.

Confocal imaging was performed in selected tissues showing pSTAT3 co-localization with GFP and DAPI, using the Nikon A1R Multi-photon confocal microscope with 488 (GFP; HV: 121; Offset: -17; Laser: 5.0), 561 (pSTAT3; HV: 111; Offset: -18; Laser: 4.5) and 405 (DAPI; HV:130; Offset: -11; Laser: 5.5) diode lasers. Under the 40x objective, z-stacks (1024x1024; 0.31 µm pixels, optical resolution: 0.18 µm) of 0.55 µm steps (20 steps in total; pinhole at 1 AU) were taken using NIS-Elements AR 4.00.00 software (Nikon® Instruments Inc.).

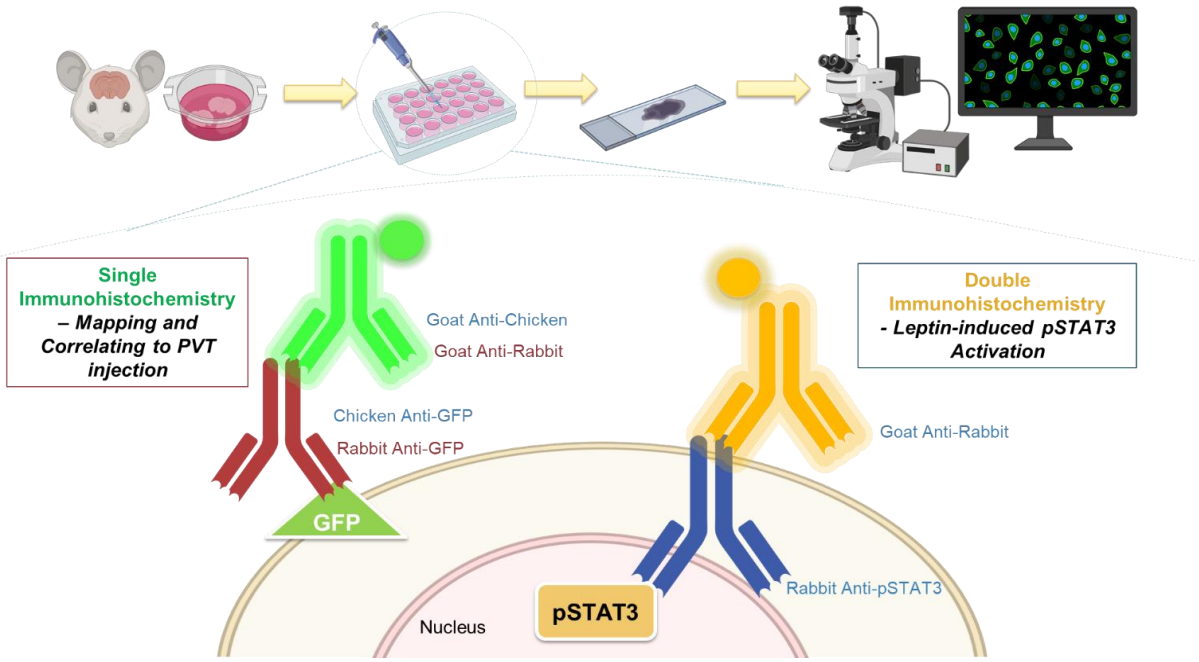

Figure 2.2 Summary of histological processes. PVT: paraventricular nucleus of the thalamus. GFP: green fluorescent protein. pSTAT3: phosphorylated signal transducer and activator of transcription 3.

2.3 Analysis

Images were analysed using ImageJ (1.52a; 64-bit) and Inkscape 0.92.4. Mapping of retrogradely-labelled GFP cell bodies in the established regions of ARC (medial, lateral or dorsal) across different anteroposterior levels and correlation of the cell locations with PVT injection site was identified with the aid of a stereotaxic rat atlas (Paxinos and Watson, 2009). Key structures, i.e. ARC, and PVT, were identified on stained sections, by firstly deciding which AP level of the section best correlated with the atlas, followed by overlaying them and scaling the image or the section to achieve the best fit. Green staining, indicating GFP, in the PVT was an indirect measurement of the injection site (Figure 2.3).

With the assistance of ImageJ, I optically analysed 1) the intensity and quantification (using multi-point tool) of pSTAT3 immunostaining of nuclei 2) quantification of neurons expressing

GFP in the ARC and 3) delineation of boundaries of intensely stained regions indicating the injection site in PVT.

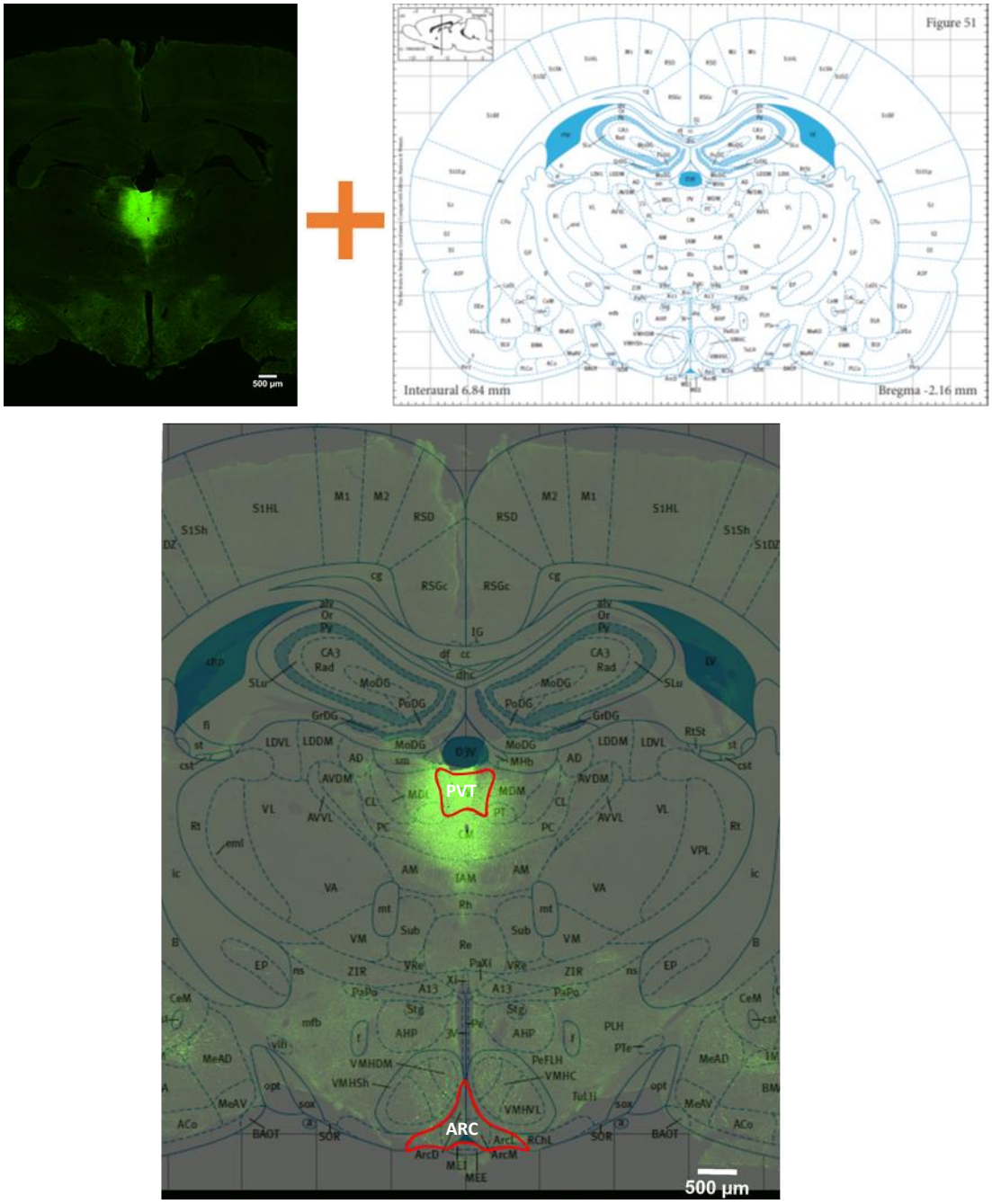

Figure 2.3 Identifying nuclear boundaries for the mapping study. An example of a GFP overview image of a tissue section overlaid and scaled with corresponding rat stereotaxic atlas (Paxinos and Watson, 2009) for analysis. Red lines drawn outline PVT (paraventricular nucleus of thalamus) and ARC (arcuate nucleus of hypothalamus) regions on the section. Scale Bar = 500 µm.

Distributions of GFP-expressing cells co-localized with pSTAT3 in leptin-treated animals were compared between observed and expected distribution using the Chi-square test (GraphPad Prism 8.2.1). Statistical significance was considered at $p<0.05$.

3. Results

3.1 Pilot Experiment: GFP Enhancement

Prior to anatomical characterization experiments, a pilot experiment (n = 4 brain sections from 1 animal) was performed to see if the endogenous fluorescent signal from GFP in neurons transfected with the virus was strong enough to be visualised. Tissue was processed either for single-label GPF enhancement or to visualise endogenous GFP. Results showed that the endogenous GFP signal (no primary antibody for GFP; Figure 3.1 A and B) revealed no apparent GFP staining in the ARC and PVT. In contrast, using the same luminescence parameters (GFP laser power: 0.5%. DAPI laser power: 33.8%) on the confocal microscope, with GFP enhancement (with primary antibody for GFP) an intense and clear staining of the PVT injection site as well as fibre and cell body staining in the ARC was visualised (Figure 3.1 C and D). From these observations, I concluded that GFP enhancement was needed for upcoming experiments in the project.

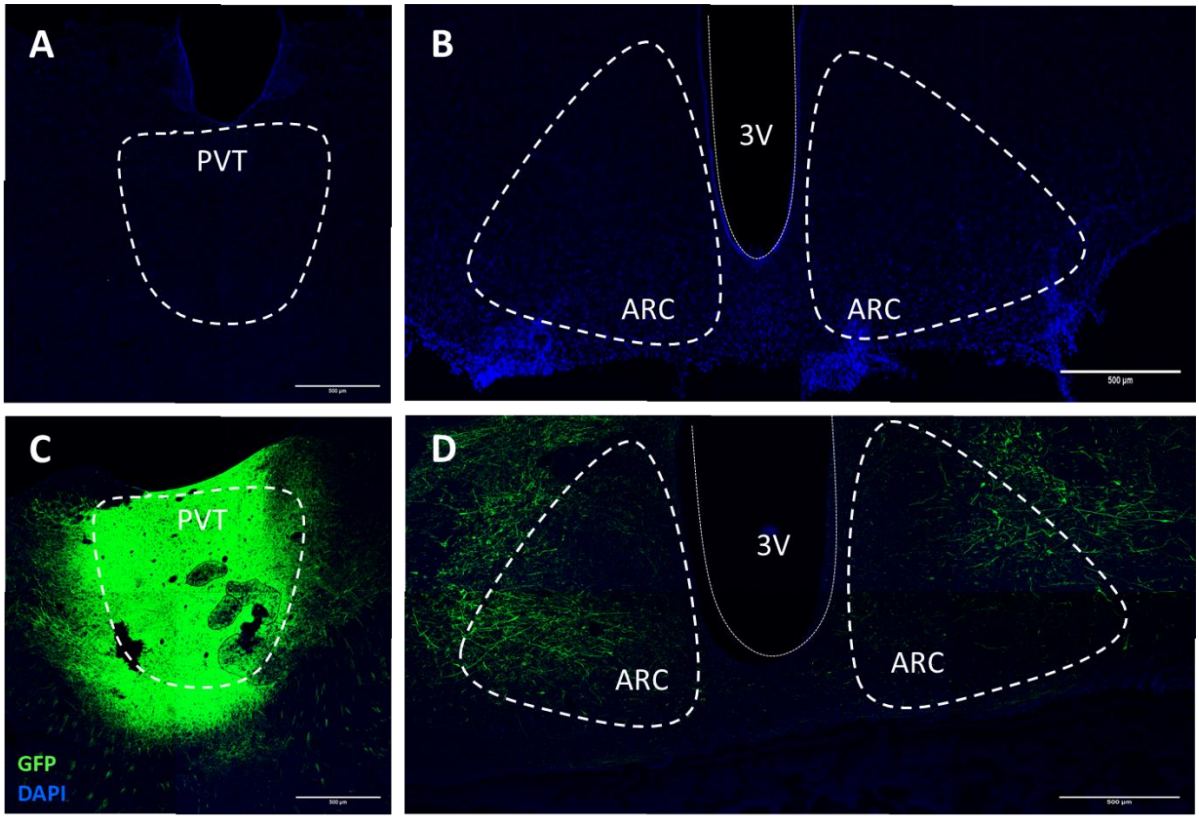

Figure 3.1 Single GFP immunohistochemical staining for enhancement pilot experiment. Endogenous GFP signal (no primary antibody) showed no apparent staining in the PVT (**A**) and ARC (**B**). With GFP enhancement using primary antibody against GFP combined with secondary fluorescent antibody (green), intense staining was seen in PVT (**C**) and ARC (**D**). Blue staining is DAPI, included in the mounting medium. PVT: paraventricular nucleus of thalamus. ARC: hypothalamic arcuate nucleus. 3V: third ventricle. Scale bars = 500 μm.

3.2 Mapping Retrogradely-Labelled GFP Cells in ARC

To address the first and second objectives of detecting and mapping the locations of ARC neurons that project to the PVT, single-label immunohistochemistry for GFP was performed (n = 9 animals). After fluorescent microscopy analysis and overlaying image of stained slices with their corresponding stereotaxic rat brain atlas sections (Figure 3.2 A), results showed that retrogradely-labelled ARC cell bodies were expressed along the anterior-posterior (AP) axis of the ARC.

Figure 3.2 B shows three examples of GFP-labelled cells found in one rat. These neurons occur at different AP levels and are distributed across different zones of the ARC. Examples from another rat are shown in Figure 3.2 C. Again, these neurons occur at different AP levels and are distributed across medial, lateral and dorsal zones of the ARC. These typical sections suggest that retrogradely-labelled cells can be found in all three anatomical zones of ARC.

This was confirmed by pooling all identified cells from all animals, shown in Figure 3.3. GFP-expressing cells were clearly distributed into all zones, across all AP levels of ARC. In all 9 animals, these retrogradely-labelled GFP cells were found located in the medial, lateral and dorsal ARC regions.

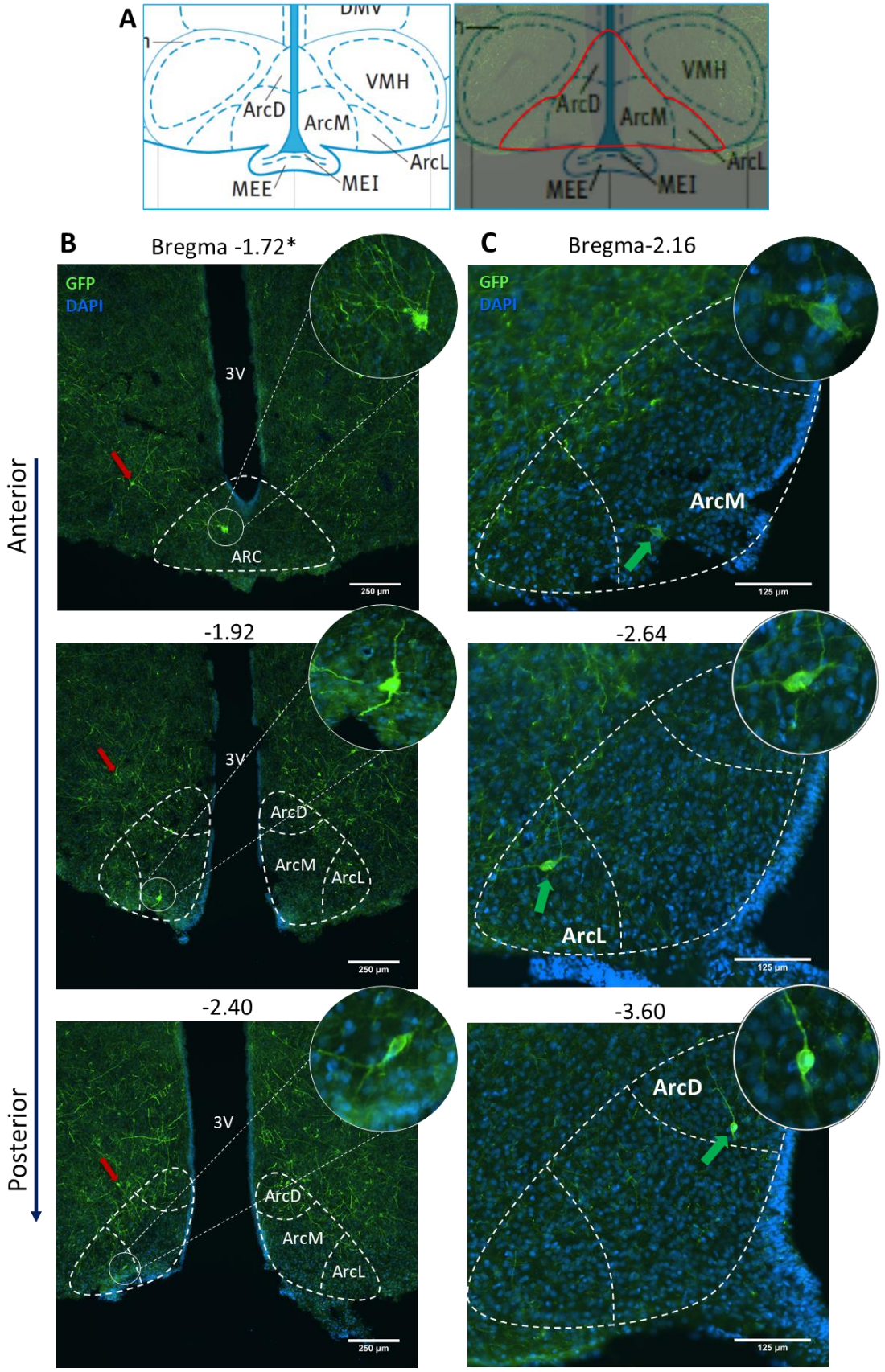

Figure 3.2 GFP immunostaining of retrogradely-labelled cell bodies in the ARC. **A)** Representative coronal section of rat atlas showing ARC and associated regions. Red line outlines the ARC region on the stained section corresponding to atlas (Paxinos and Watson,

2009). **B)** GFP-labelled (green) neurons found in the ARC at different anteroposterior levels in one rat (10x objective; scale bars = 250 μm). Blue: DAPI nuclear staining. *At bregma -1.72, ARC has not split into medial, lateral and dorsal regions. Red arrows indicate cells located outside the ARC region. **C)** GFP-labelled neurons found in different regions in the ARC in another rat (20x objective; scale bars = 125 μm). ArcM: medial ARC. ArcL: lateral ARC. ArcD: dorsal ARC. 3V: third ventricle.

GFP-labelled neurons, indicating neurons that project into the PVT, were located across several hypothalamic nuclei. This can be seen in Figure 3.2 B where several GFP-labelled cells are found scattered beyond ARC borders, across different AP levels (indicated by the red arrows). For the purpose of the study, I focused on the ARC. Mapping every GFP-labelled cell in the ARC (n = 150 neurons from 9 animals; range: 4 – 38 neurons per animal) revealed that these retrogradely-labelled neurons that projected to the PVT were distributed in the great majority within the medial (n = 74 neurons) and lateral (n = 61 neurons) ARC, with some in the dorsal ARC (n = 15 neurons) (Figure 3.3 A and B). These immunohistochemical analyses confirmed the presence of neurons within the ARC that sends projections to the PVT.

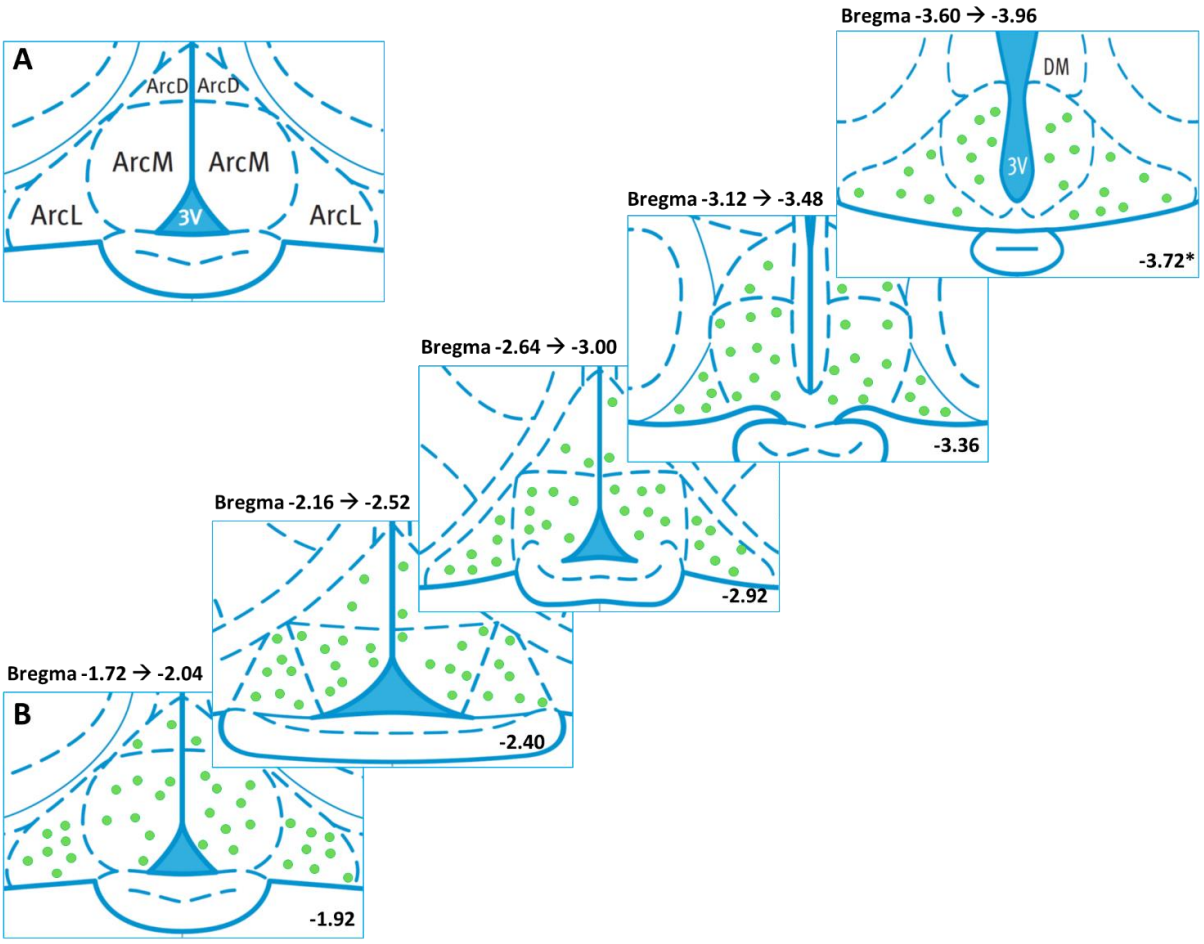

Figure 3.3 Summary of anatomical mapping of GFP-labelled ARC neurons. A) Schematic of ARC regions from coronal view of rat brain atlas indicating labels applying to all of B as well. ArcL: lateral ARC; ArcM: medial ARC; ArcD: dorsal ARC; 3V: third ventricle (modified from Paxinos and Watson, 2009). **B)** Distribution of all GFP-labelled ARC neurons found (n = 150 neurons from 9 animals) along the anteroposterior axis (4 brain sections per atlas level). Green dots represent GFP-labelled ARC cell bodies. *At bregma -3.72, the dorsal ARC is no longer present. DM: dorsomedial hypothalamic nucleus.

3.3 Correlating Cell Locations to PVT Injection Site

I then addressed my third objective and examined whether the expression of the GFP-labelled cells in the ARC correlated with the virus injection site or spread in the PVT. Qualitative assessment of green staining of GFP in the PVT was an indirect measure of the injection site.

From fluorescent microscopy imaging of single GFP immunostaining and rat brain atlas-assisted mapping (Figure 3.4 A), it was revealed that the expression of retrogradely-labelled

cells in the ARC occurred only when there was an intense and centered staining including the PVT (Figure 3.4 B; in 9 out of 11 animals). In contrast, an off-target PVT injection (Figure 3.4 C) or a less intense and off-centred targeting of the PVT correlated with the absence of GFP-labelled cells in the ARC (found in 2 out of 11 animals).

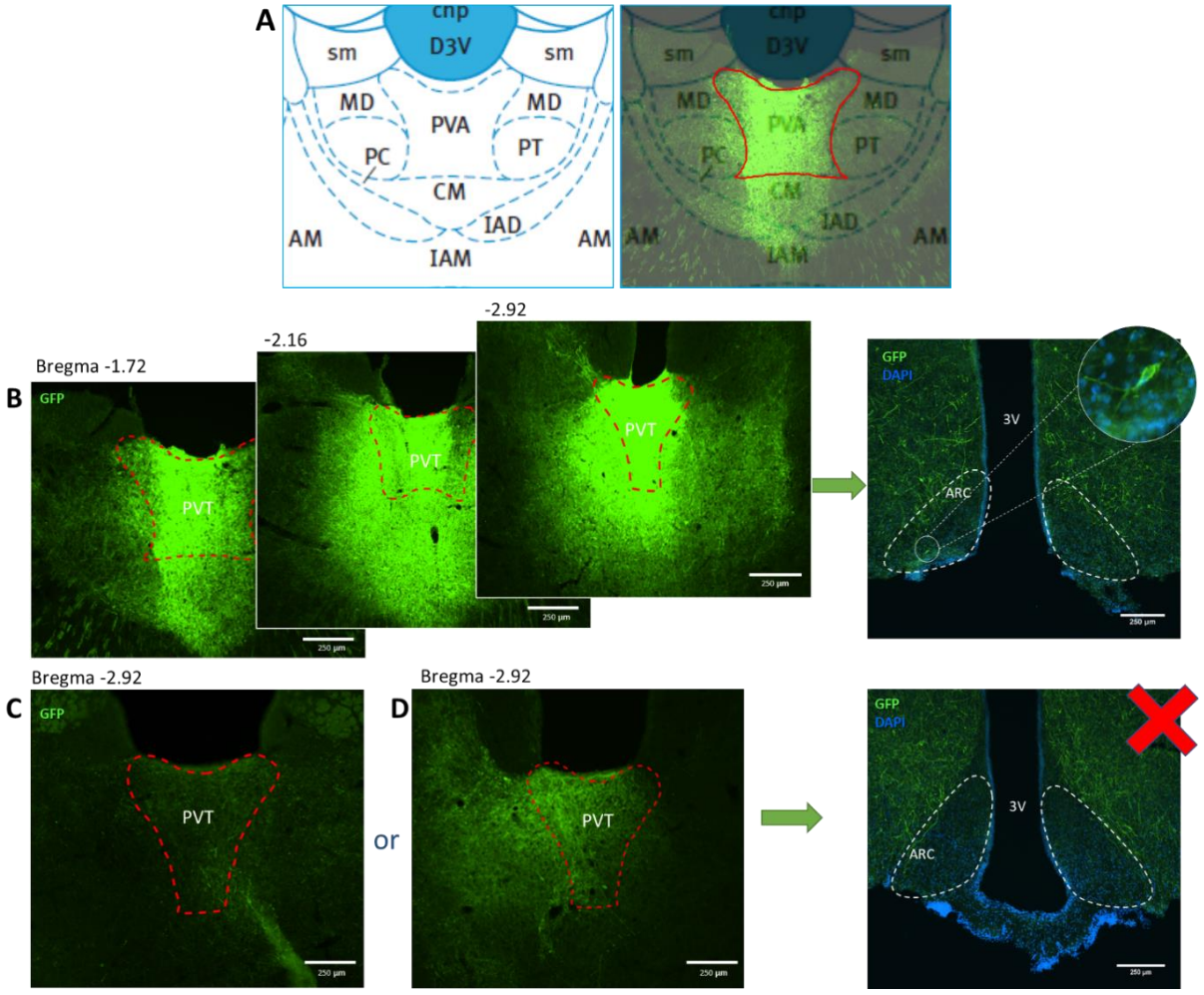

Figure 3.4 Immunostaining of GFP-expressing virus injections in the PVT. A) Representative coronal view of rat brain atlas showing the PVT and associated regions. Red line outlines the PVT region on stained section overlaid with corresponding atlas (Paxinos and Watson, 2009). **B**) Intense and centred GFP staining including the PVT correlated with presence of retrogradely-labelled neuron in ARC. **C**) Off-target injection or **D**) those injections not centred and less intensely stained correlated with absence of GFP-labelled neurons in the ARC. PVT: paraventricular nucleus of thalamus. ARC: arcuate nucleus of hypothalamus. 3V: third ventricle. Scale bars = 250 µm

3.4 Leptin-Induced Activation of pSTAT3 in the ARC

To address the fourth objective of investigating whether ARC neurons that projected to the PVT respond to leptin, double immunohistochemistry was performed for GFP and pSTAT3, a downstream signaling activation marker for leptin. Firstly, a series of optimisation experiments for the double-labelling were performed to eliminate non-specific binding of the secondary antibody. I selected the antibody dilution (1:500) and the antigen retrieval method (submerging in water bath for 90°C) that produced the optimal signal: noise ratio.

Using the optimised protocol, microscope analysis revealed that the negative control experiment (no primary antibodies) showed only DAPI expression with no GFP cell or pSTAT3 nuclei staining in the ARC, confirming the absence of unspecific binding for the secondary antibodies (Figure 3.5 A).

In food-restricted and leptin-injected rats, dense pSTAT3 nuclei staining in the ARC was seen as illustrated by the example in Figure 3.5 B. All nuclei with obvious staining were quantified from two sections in each of five animals. On average, there were 108.5 pSTAT3-positive nuclei per section (from n = 5 animals; range: 66 – 147 nuclei). In contrast to this, in one food-restricted but non-leptin-injected control animal, while there were pSTAT3-positive nuclei, the staining was visibly fainter (Figure 3.5 C). In addition, there was a much lower count of 35 nuclei (from n = 1 animal). It is well known that pSTAT3 is low without leptin challenge (Maniscalco and Rinaman, 2014). This control animal ensured that with my protocol, a low level of pSTAT3 labelling was visualised in an unchallenged animal compared to leptin-challenged animals to identify responsive cells. Hence, these control experiments confirmed that leptin administration led to the upregulation of pSTAT3 in the ARC, superimposed on a low level of constitutive expression.

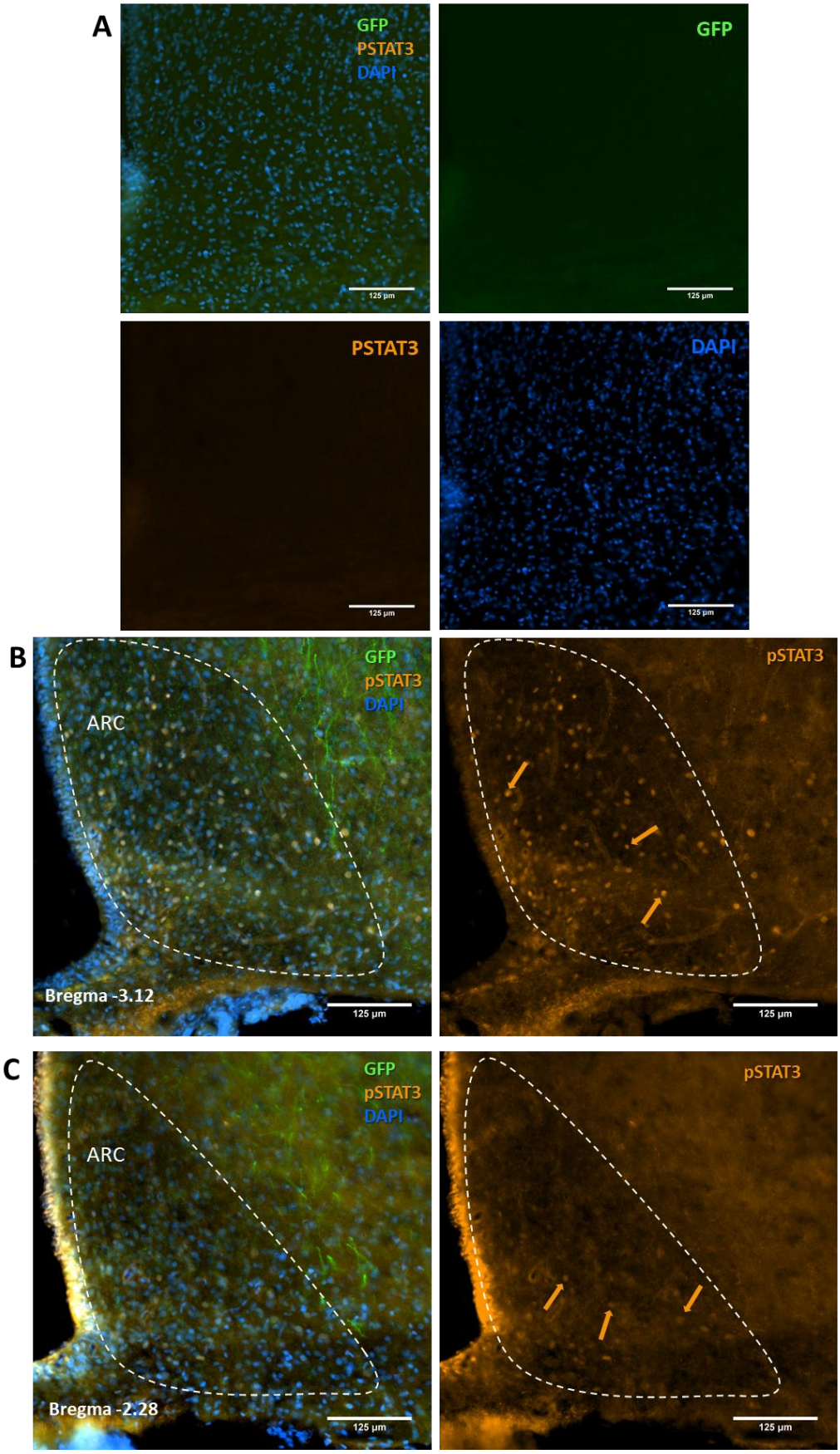

Figure 3.5 Control immunostaining experiments for leptin group. A) Negative control for pSTAT3 and GFP double immunostaining. Representative images showing merged, GFP neuron (green), pSTAT3 nuclei (orange) and DAPI nuclei (blue) expression. **B)** Food-restricted

and leptin-injected animals. ARC showing clear and numerous staining of pSTAT3 nuclei. Orange arrows indicate examples of pSTAT3 nuclei. **C)** Control animal on food restriction without leptin injection. ARC showing fainter and less pSTAT3 nuclei staining. Scale bars = 125 μm.

3.5 Leptin Activation of ARC-PVT Neurons

In leptin-injected animals, double immunohistochemical analysis for GFP and pSTAT3 was used to investigate whether leptin was able to activate ARC neurons that project to the PVT. Of all the GFP-labelled cells mapped in Figure 3.3, a total of 64 neurons were quantified from animals injected with leptin. These were available for analysis of co-localization of GFP-labelled ARC neurons with pSTAT3 nuclei and DAPI nuclei staining. Of these 64 neurons, 21 showed co-localization with pSTAT3 (from n = 5 animals), indicating that a sizable proportion (33%) of ARC neurons that project to the PVT respond to leptin. Figure 3.6 A and B show examples of the co-localization of GFP-labelled ARC neurons with pSTAT3 and DAPI nuclei staining. Some pSTAT3 nuclei were not co-localized with a GFP cell, indicating that these nuclei belonged to different cells which did not project to the PVT.

To confirm that co-localization appearance was due to specific intracellular inclusion of pSTAT3 nuclei in GFP-labelled cells, confocal imaging and z-stack analysis was performed on a subset of double-labelled cells. As shown in Figure 3.7, these cells showed that the pSTAT3 nuclei were absent in the top and bottom sections of the z-stack (Figure 3.7 A and C), whereas it was present in the middle part of the stack (Figure 3.7 B). The top, middle and bottom part of the z-stack were 3 scans apart with each scan of the z-stack being 0.55 μm thick. These findings thus confirmed that pSTAT3 nuclei was indeed co-localized within the GFP-labelled ARC-PVT neuron.

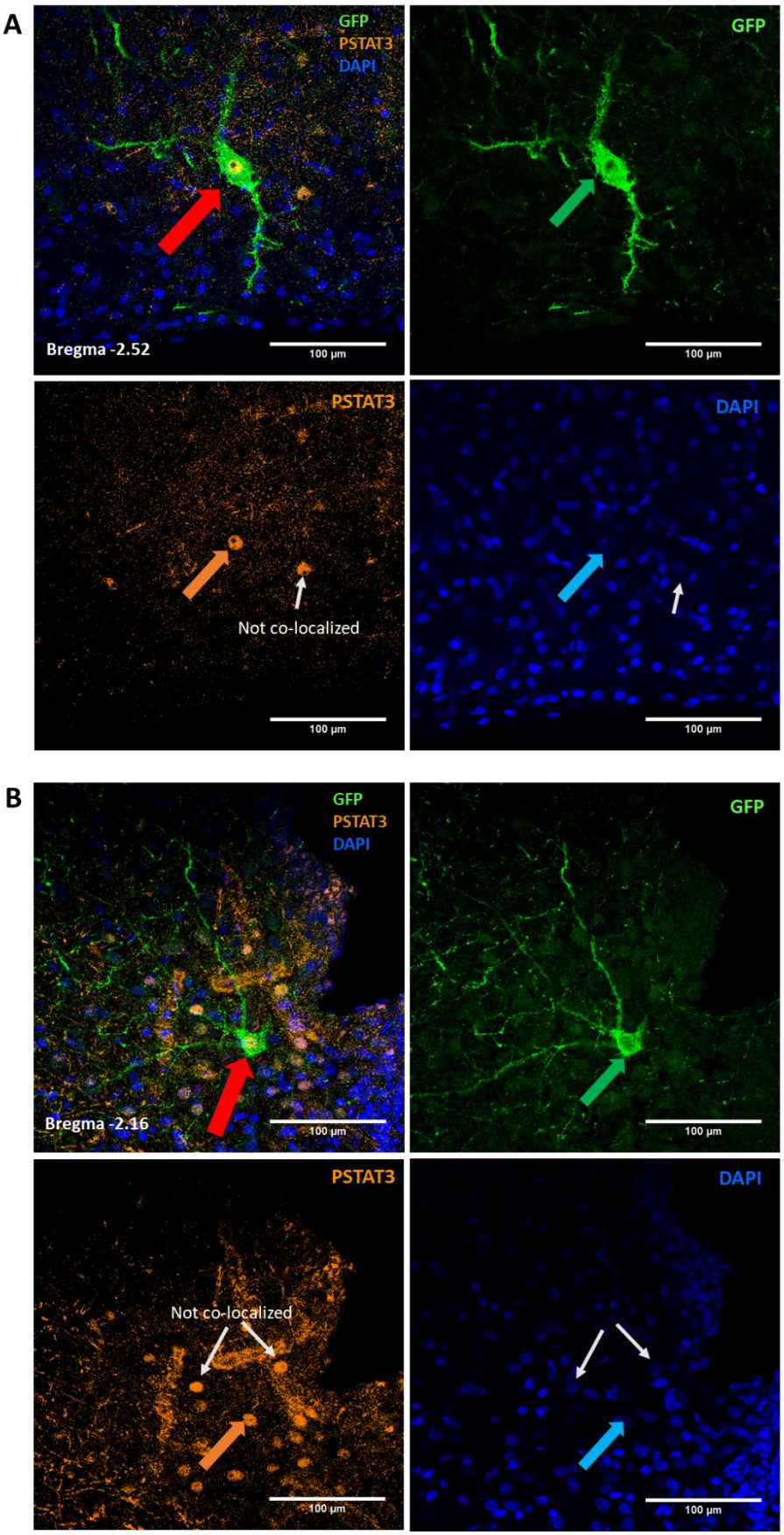

Figure 3.6 Co-localization of ARC-PVT neurons with pSTAT3 nuclei. A and **B**) Representative images from two different animals showing retrogradely-labelled ARC neurons (green) with pSTAT3 (orange) and DAPI (blue) nuclei co-localization (red arrows). Green arrows indicate GFP-labelled neuron, orange arrows show pSTAT3 stained nuclei and blue arrows indicate DAPI nuclei staining. White arrows indicate pSTAT3 and DAPI nuclei non-colocalized with GFP. Scale bars = 100 µm.

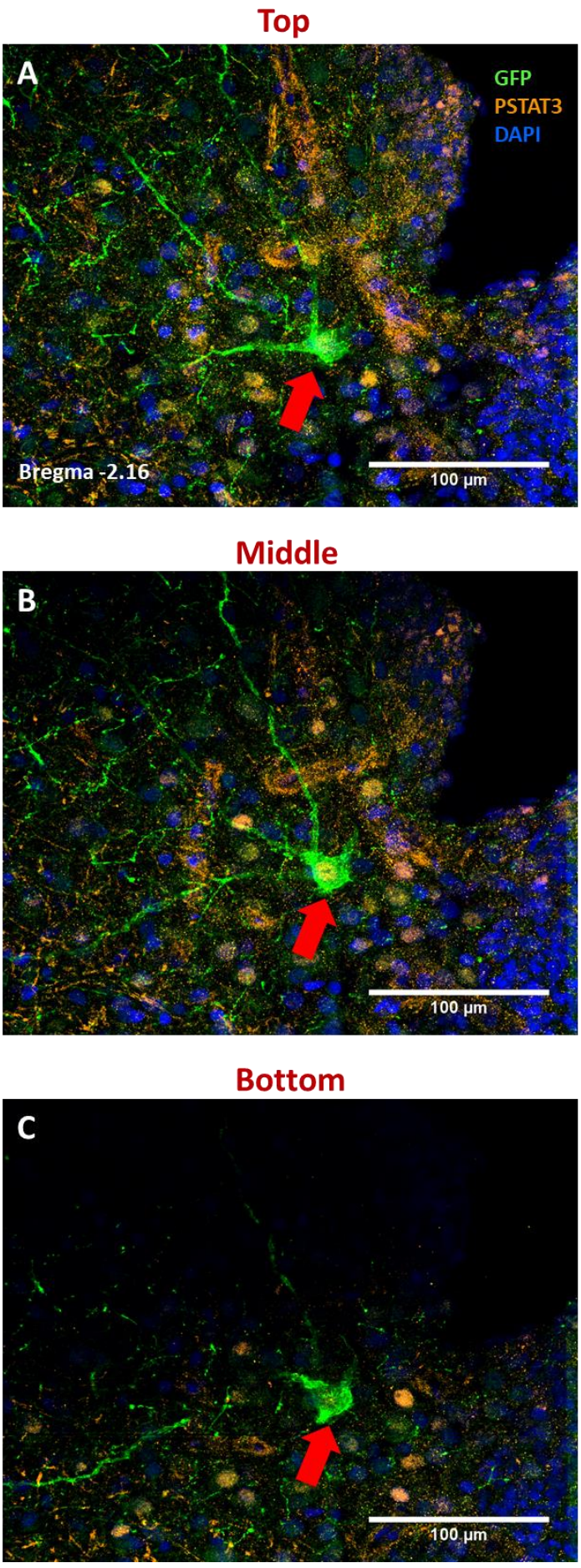

Figure 3.7 Representative images of z-stack analysis of a GFP cell with pSTAT3 nuclei co-localization. The top **(A)** and bottom **(C)** of z-stack shows GFP-labelled neuron only. The middle **(B)** section of the z-stack shows co-localization of GFP cell and pSTAT3 nuclei. Scale bars = 100 µm.

From the 64 GFP-labelled cells found in leptin-treated animals, 32 were in the medial ARC, 26 in the lateral ARC and 6 were in the dorsal ARC. The expected distribution of co-localized GFP cells with pSTAT3 were 10 in the medial ARC (48%), 9 in the lateral ARC (43%) and 2 in the dorsal ARC (9%). However, interestingly, as shown in the representative coronal section of the ARC in Figure 3.8, the observed co-labelled cells were distributed only in the medial (n = 15 cells) and lateral ARC (n = 6 cells), completely excluding the dorsal ARC. After performing a statistical analysis comparing the observed distribution of co-localized cells with the expected distribution using a Chi-square test, the distribution was not shown to be significantly different ($p = 0.064$). However, due to the low numbers, the results needed to be interpreted with caution. I would need to obtain a higher number of neurons in data for a definitive conclusion.

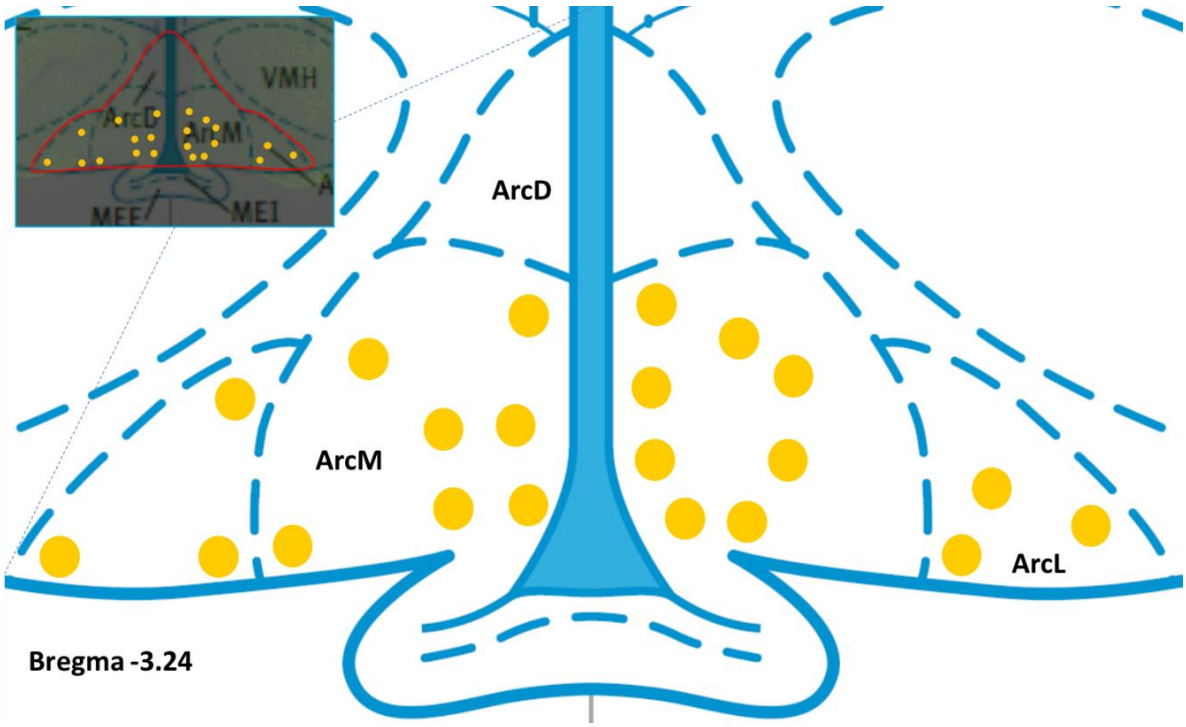

Figure 3.8 Summary of ARC-PVT neuron co-localization with leptin-induced pSTAT3 activation. A total of 21 pSTAT3 and GFP co-localized cells (yellow dots, n = 5 animals) seen in the medial (ArcM, n = 15) and lateral (ArcL, n = 6), completely excluding the dorsal (ArcD) ARC.

4. Discussion

Projections from the ARC to the PVT may have a role in the integration of homeostatic and non-homeostatic regulations of food intake. This study investigated the anatomical characteristics and leptin-hormone-responsiveness of ARC-PVT projections. The specific objectives were 1) to confirm the presence of PVT projecting neurons in the ARC, 2) to map the location of these neurons relative to established ARC subdivisions i.e. medial, lateral or dorsal ARC, 3) to examine the relation between PVT injection spread and the expression of these retrogradely-labelled neurons in ARC, and finally, 4) to investigate whether these neurons respond to the key metabolic hormone leptin, by the activation of its downstream signalling marker i.e. phosphorylated STAT3.

4.1 ARC Efferents to the PVT

To characterise the projection of ARC neurons to the PVT, a GFP-expressing retrograde virus was injected in the PVT of Wistar rats. Immunohistochemical analysis and atlas-assisted mapping showed retrogradely-labelled GFP cells in the ARC (Figure 4.1). These GFP-labelled cells were distributed in the great majority in the medial and lateral ARC with some in the dorsal ARC. Additionally, the presence of these retrogradely-labelled cells in the ARC did not occur if there was poor expression of virally induced GFP in the PVT, or if the injection was not centred in PVT. These observations confirmed the presence of neurons in the ARC which send projections to the PVT. This can be supported by previous anterograde and retrograde tracing experiments as well as immunocytochemistry labelling experiments, which showed projections of neurons originating from the medial and lateral ARC to the PVT (Atasoy et al., 2012; Kirouac et al., 2015; Lee et al., 2015; Parsons et al., 2006; Sim and Joseph, 1991; Wang et al., 2015).

The ARC, located at the base of the hypothalamus, has been implicated as the primary hypothalamic node that integrates peripheral signals and central neuropeptides, which modulate homeostatic feeding and energy expenditure (Chronwall, 1985; Matafome and Seica, 2017; Wynne et al., 2005). On the other hand, the PVT, located underneath the third ventricle, has been suggested as a relay station in energy balance and food reward (Kirouac, 2015; Lee et al., 2015). Thus, the ARC to PVT connection could potentially be important in linking energy balance and reward.

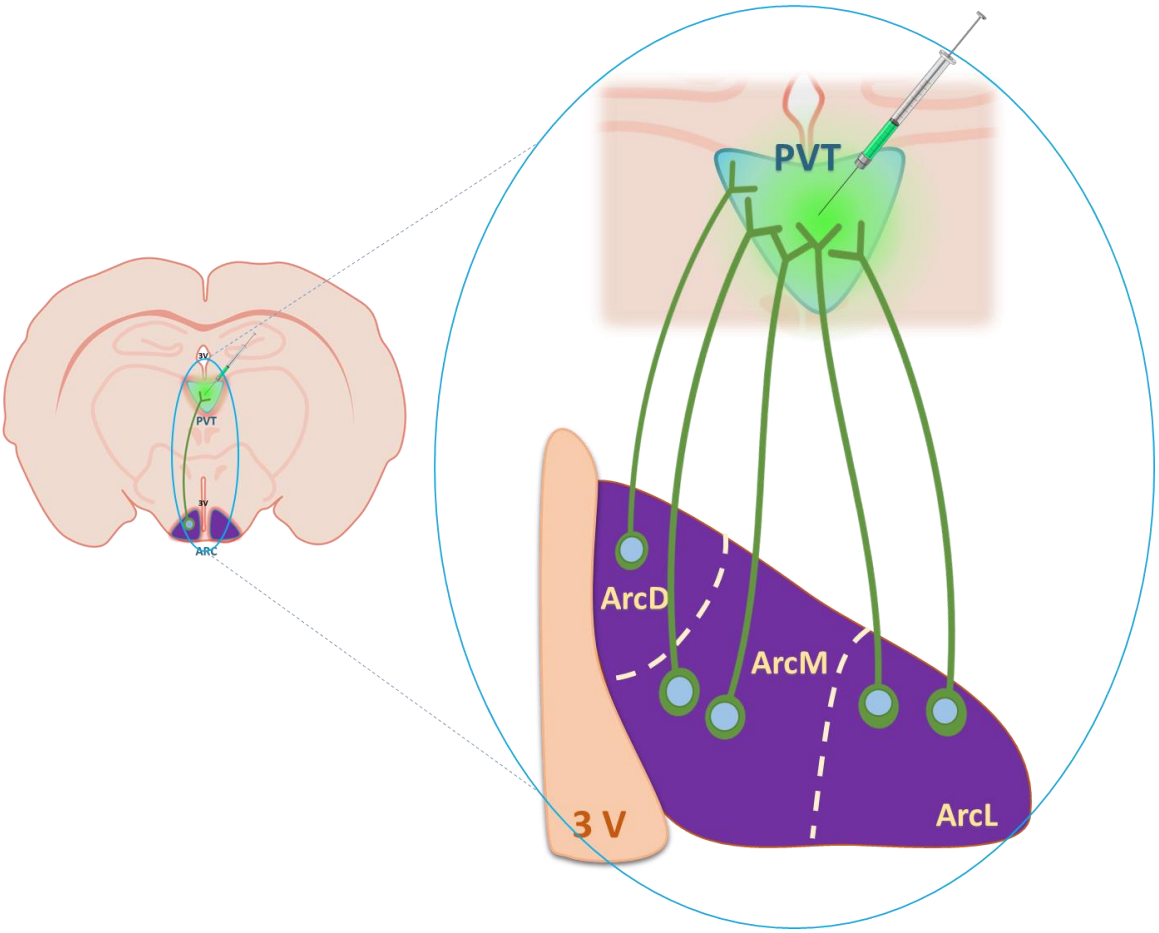

Figure 4.1 Schematic representation of retrogradely-labelled ARC neurons projecting to PVT found in the current study. Injection of GFP-expressing virus in the PVT led to the expression of GFP in neurons with terminals in the PVT. ArcD: dorsal ARC. ArcM: medial ARC. ArcL: Lateral ARC. PVT: paraventricular nucleus of thalamus. 3V: third ventricle.

4.2 Leptin Activates pSTAT3 in the ARC

To examine whether the ARC is responsive to the key metabolic hormone leptin, double-labelling immunohistochemistry was performed for GFP and pSTAT3, a known downstream activation marker of leptin signalling. Fluorescent microscopy analysis revealed that in food-restricted and leptin-injected animals, numerous dense pSTAT3-positive nuclei staining was seen. In comparison to this, the non-leptin-injected control animal, which determined the baseline pSTAT3 activation of ARC neurons in fasted rats, and should have a very low intrinsic leptin level, expressed fainter and lower number of pSTAT3-positive nuclei in the ARC. This strong difference in intensity and quantity of pSTAT3 nuclei expression between administered and intrinsic leptin animals confirmed that leptin injection induces the activation of pSTAT3 nuclei in the ARC. These findings can be supported by previous *in vivo* and *in vitro* studies where leptin signalling was shown to have physiological effects in the ARC (Cabanelas et al., 2006; Dardeno et al., 2010; Kim et al., 2000). Furthermore, the administration of leptin in rats have also been shown to increase levels of STAT3 phosphorylation in the ARC (Munzberg et al., 2003).

The ARC has been considered the hypothalamic area that primarily senses circulating peripheral hormones, namely from gut and adipose tissue, and nutrient signals. (Matafome and Seica, 2017). It lies adjacent to the third ventricle and immediately above the median eminence, a region where the blood-brain barrier is specially modified to allow peripheral peptides, like the anorexigenic metabolic hormone leptin, access to receptors and influence energy homeostasis (Dardeno et al., 2010; Wynne et al., 2005).

4.3 Co-localization of pSTAT3 and GFP in ARC neurons

To investigate whether ARC neurons projecting to the PVT are leptin-sensitive, double-labelling immunohistochemistry was performed for GFP and pSTAT3. The double-labelling immunohistochemical analysis revealed co-localization of pSTAT3 nuclei with GFP neurons in the ARC. This intranuclear inclusion of pSTAT3-positive nuclei within GFP-labelled cells was confirmed with z-stack analysis. Approximately 1/3 of retrogradely-labelled GFP cells in the ARC showed co-localization with pSTAT3, indicating that a sizable proportion of ARC neurons that project to the PVT respond to leptin (Figure 3.7 and 3.8) Additionally, when mapping the locations of these leptin-activated neurons in the ARC, they were found distributed only in the medial and lateral ARC, completely excluding the dorsal ARC (Figure 3.8 and 4.2). However, with the number of neurons tested, only 2 neurons might have been expected to be in the dorsal ARC, and so more work would be needed to confirm this.

Several leptin-sensitive neuronal populations with opposing effects on food intake have been revealed to play key roles in the ARC (Cone et al., 2001). One neuronal circuit inhibits food intake, via the expression of the neuropeptides POMC and CART in the lateral ARC. The other neuronal circuit stimulates food intake, via the expression of NPY and AgRP neuropeptides (Suzuki et al., 2010; Wynne et al., 2005). Previous electrophysiological studies have demonstrated that these leptin-responsive populations project to the PVN and the LH (Cone et al., 2005; Elias et al., 1999). However, the response of ARC-PVT projections to leptin has not been explored. Together, my results confirmed my hypothesis and is the first study that demonstrated the anatomical and functional characterisation of a leptin-activated neuronal population in the ARC with projections to the PVT.

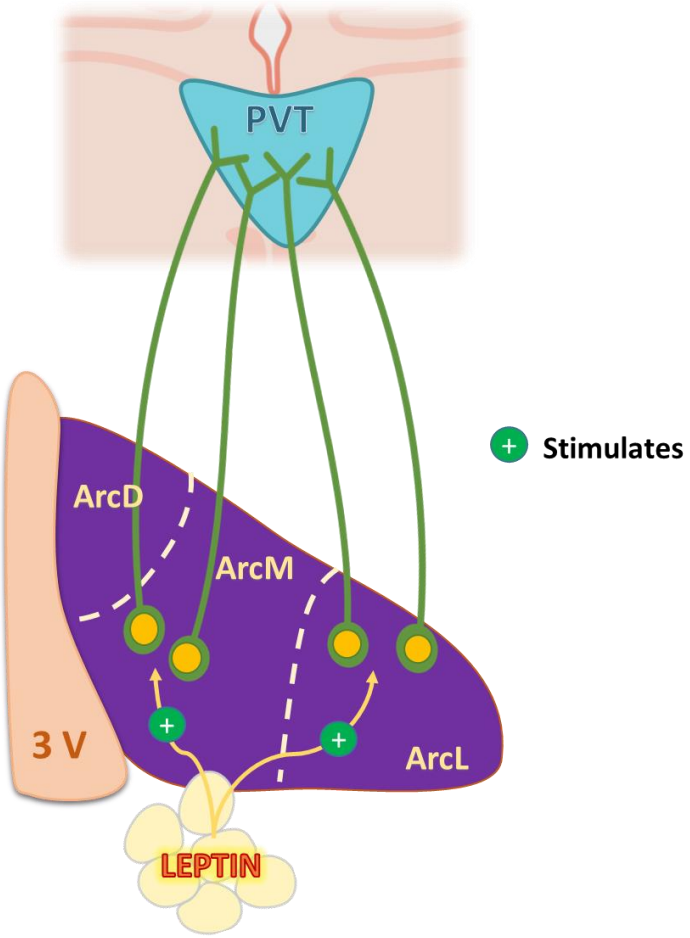

Figure 4.2 Schematic representation of leptin activation of ARC-PVT neurons found in the current study. Results showed that leptin stimulated (+) GFP-labelled ARC neurons that project to the PVT. These neurons were distributed in the medial (ArcM) and lateral (ArcL) only, excluding the dorsal ARC (ArcD). Yellow nuclei indicate leptin-induced pSTAT3 activation. 3V: third ventricle.

4.4 Implications of ARC-PVT Connection in Food Intake

The ARC is a brain region shown to be critical in the homeostatic regulation of food intake, involving physiological responses and ingestion of food according to metabolic state, i.e. hungry or satiated (Baver et al., 2014). Leptin, which diffuses through the blood-brain-barrier and acts via the JAK2-STAT3 signalling pathway, have been shown to stimulate anorexigenic POMC/CART co-expressing neurons located in the lateral ARC. Leptin conversely inhibits the orexigenic NPY/AgRP co-expressing neurons located in the medial ARC. The opposing effects

of leptin lead to the overall depression of appetite and weight loss (Chen, 2016; Liu and Kanoski, 2018; Monteleone and Maj, 2013; Yu and Kim, 2012). These different neuronal populations have been shown to project to the PVN and the LH (Figure 4.3; Chen, 2016; Schwartz et al., 2000; Yu and Kim, 2012).

Additionally, supporting the findings in this study, previous optogenetic stimulation and tracing experiments have revealed that the different neuronal populations in the ARC also project to the PVT and have been implicated in the regulation of food intake (Atasoy et al., 2012; Betley et al., 2013; Kirouac et al., 2006; Lee et al., 2015; Parsons et al., 2006). Thus, it is fair to speculate that the leptin-activated ARC-PVT neuron in the lateral ARC identified in this study could perhaps be POMC/CART neurons (Figure 4.3). However, further studies would need to be conducted in order to confirm this phenotype.

The potential neurochemical phenotype of the retrogradely-labelled leptin-activated neurons in the medial ARC is less clear. This is because the NPY/AgRP projecting neurons are known to be inhibited by leptin (Chen, 2016; Liu and Kanoski, 2018). However, results depicted that retrogradely-labelled GFP cells in the medial ARC were also activated by leptin. This therefore eliminates the possibility of these neurons being NPY/AgRP neurons. This suggests that a distinct neuronal population exists in the medial ARC that is activated by leptin and projects to the PVT (Figure 4.3).

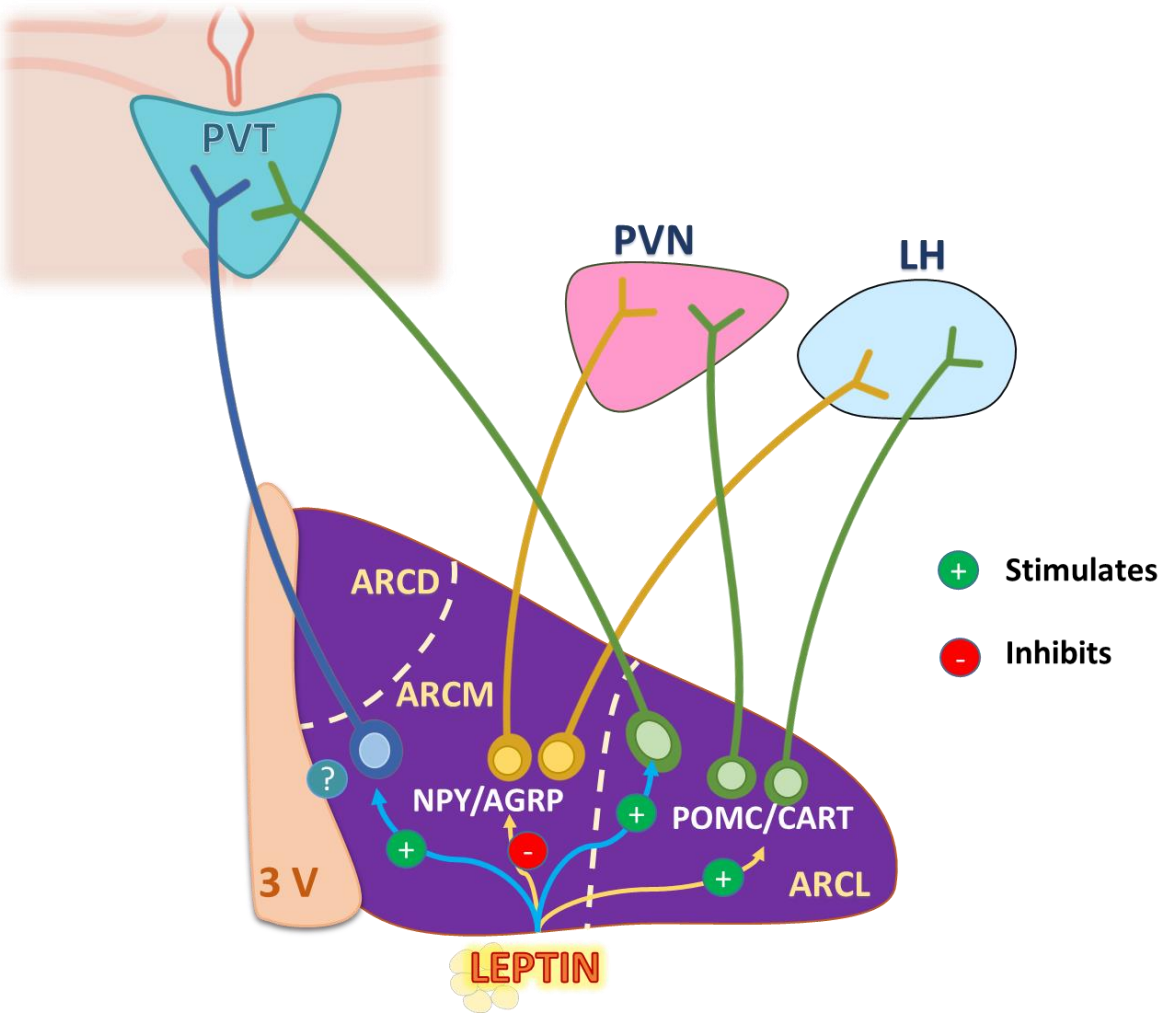

Figure 4.3 Schematic representation of the cell types and connections engaged by leptin in the ARC. Diagram shows known and new interactions of leptin with specific neural phenotypes in the arcuate nucleus (ARC) with projections to structures important for regulation of food intake. (-), (+) indicate inhibitory or stimulatory effect respectively. (?) indicate unknown neuronal phenotype. POMC/CART: proopiomelanocortin and cocaine-and-amphetamine-regulated transcript co-expressing neurons. NPY/AgRP: neuropeptide Y and agouti-related peptide co-expressing neurons. PVN: paraventricular nucleus of the hypothalamus. LH: lateral hypothalamus. PVT: paraventricular nucleus of the thalamus. 3V: third ventricle.

4.5 ARC-PVT Connection: A Potential Circuit Integrating Metabolic State and Reward

Our metabolic state, i.e. hungry or satiated, has been shown to influence eating behaviour by modulating the hedonic value of food and food-associated cues (Toates, 1986). This reward-associated behaviour is thought to be mediated by neurochemical crosstalk between homeostatic and non-homeostatic systems (Berthoud, 2006; Berthoud et al., 2017). The present study investigated a potential circuit linking the two systems. The study showed that the metabolic action of leptin in the ARC is transmitted to the PVT (Figure 4.4).

The PVT, located in the dorsal midline of the thalamus has been implicated as a critical component in the feeding inputs to the neural circuitry involved in incentive salience and behavioural responses to reward-associated cues (Haight et al., 2017; Lee et al., 2015). This can be supported by previous electrophysiological and retrograde tracing studies which demonstrated the association of the PVT with the mesolimbic DA reward pathway (Li and Kirouac, 2008; Parsons et al., 2007; Perez and Lodge, 2018) The PVT relays arousal-related efferents to the nucleus accumbens (NAc), a major recipient of the DA signals from the VTA, known to mediate arousal, and reward-associated behaviours (Kirouac, 2015; Parsons et al., 2006; Figure 4.4).

Additionally, PVT neurons projecting to the NAc have also been found to receive interactive POMC/CART and NPY/AgRP terminals, originating from the lateral and medial ARC respectively (Atasoy et al., 2012; Betley et al., 2013; Lee et al., 2015; Parsons et al., 2006). The current study elucidates the potential role of ARC to PVT projections in the integration of metabolic or arousal-related inputs on their way to the mesolimbic reward pathway and may influence motivational and hedonic behaviours in feeding (Figure 4.4).

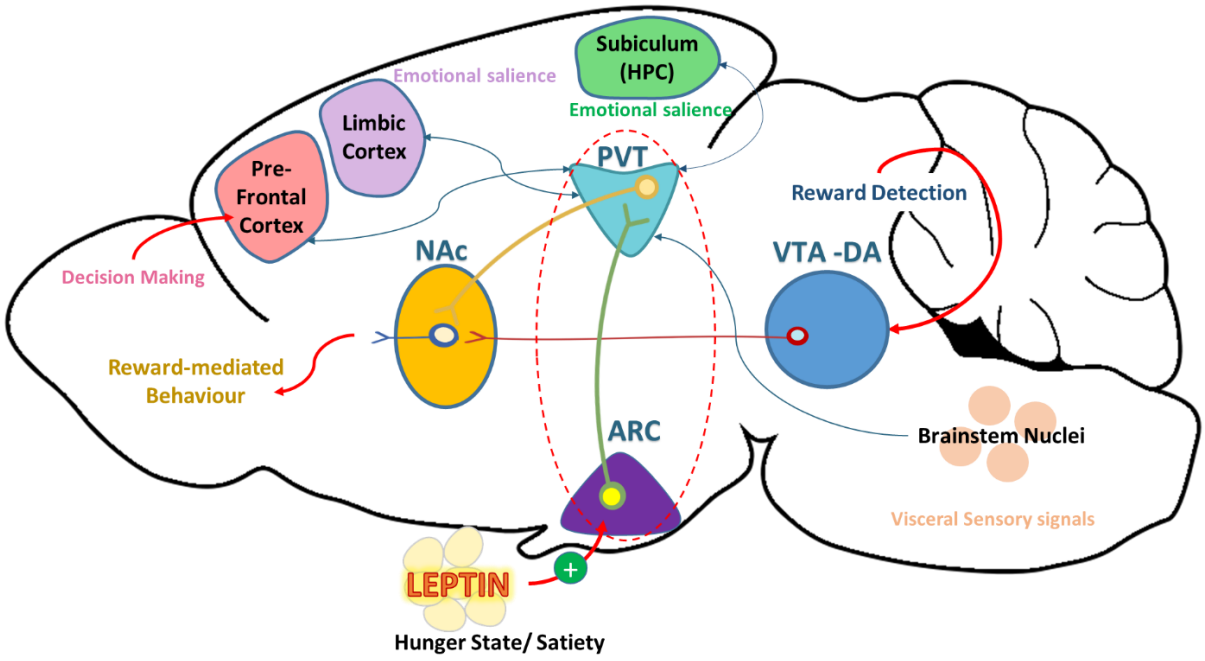

Figure 4.4 Schematic of neural nodes controlling food intake and reward. The PVT may integrate emotional saliency information from the subiculum, limbic cortex and prefrontal cortex (PFC) with viscerosensory information from the ARC and brainstem to guide behaviour. Dotted line indicates the new potentially critical circuit- the projection of ARC neurons to the PVT and its possible implications in responses to food-associated cues. (+) denotes stimulatory effects of leptin signal in ARC and its transmission to the PVT. PVT: paraventricular nucleus of thalamus. ARC: hypothalamic arcuate nucleus. NAc: nucleus accumbens. VTA: ventral tegmental area. DA: dopamine. HPC: hippocampus.

4.6 Summary and Conclusions

In this study, I identified and anatomically characterised ARC neurons that respond to leptin and send projections to the PVT. This new connection may serve as an integral part of a novel pathway that is potentially an important regulator of non-homeostatic reward-mediated behaviours in response to food cues. Future anatomical and electrophysiological investigations are needed to answer important questions to further understand the functional implications of this link between ARC and PVT. What are the specific phenotypes of these ARC-PVT neurons? Do ARC-PVT neurons project to PVT-NAc neurons? Does leptin modulate ARC-PVT neurons

that project to the reward system? Answers to these questions would go a long way in providing a better understanding of how the PVT fits within a complex circuitry that regulates non-homeostatic reward-associated behaviours in food intake. It may also provide new knowledge about how the brain integrates energy homeostasis and reward signals. Finally, it could potentially highlight implications of reward in the regulation of appetite, and in clinical conditions such as obesity and anorexia.

4.7 Future Directions

One of the limitations of this study was that I did not have an exact marker for the GFP-expressing virus injection site in the PVT, making it difficult to precisely correlate ARC neurons with PVT injection staining. For future investigations, a marker e.g. a dye or fluorescent retro-beads (Lee et al., 2015), can be used to delineate this. Furthermore, this project investigated whether there were any ARC neurons that project to the PVT. The present study was not designed to answer the question of "What is the percentage/proportion of ARC neurons that project to the PVT?". For that, stereological methods would be required.

As noted above, a key remaining question triggered by the present study is, what are the phenotypes of ARC-PVT neurons? To address this, future studies could use triple-labelling to determine the phenotype of ARC-PVT neurons (e.g. AgRP/NPY/POMC/CART + pSTAT3 + GFP). Although the present study was able to use pSTAT3 as a marker of leptin metabolic activation, the question remains whether this impacts neuronal electrical activity. Future studies could further evaluate this using electrophysiology to record the response of ARC-PVT neurons to leptin, and to see if pSTAT3 activation leads to a change in firing rate. And finally, future anatomical and electrophysiological studies can be conducted to investigate the role of ARC-PVT connection in the integration of homeostatic and non-homeostatic regulation of food intake.

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